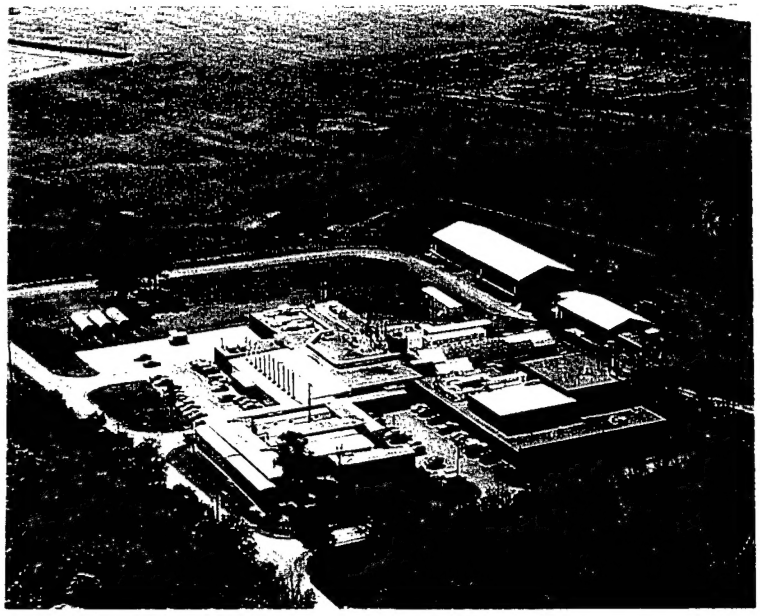
REPORT

FINAL REPORT

Task 94-33: *In Vivo* Evaluation of Temporary Wound Dressings for Adherence, Durability and Autografting on Sulfur Mustard-Induced Lesions in Weanling Swine



To

U.S. Army Medical Research Institute of Chemical Defense

December, 2000

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FINAL REPORT

Contract No. DAMD 17-89-C-9050
A Medical Research and Evaluation Facility (MREF) and Studies Supporting the Medical Chemical Defense Program

on

Task 94-33: *In Vivo* Evaluation of Temporary Wound Dressings for Adherence, Durability and Autografting on Sulfur Mustard-Induced Lesions in Weanling Swine

to

U.S. Army Medical Research Institute of Chemical Defense

November, 2000

by

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FINAL REPORT

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***In Vivo* Evaluation of Temporary Wound Dressings for Adherence, Durability and Autografting on Sulfur Mustard-Induced Lesions in Weanling Swine**

Task 94-33

December, 2000

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EXECUTIVE SUMMARY

The U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) is responsible for evaluating treatment regimens for percutaneous bis (2-chloroethyl) sulfide (sulfur mustard; SM) exposure and for developing models to make these assessments. Task 94-33 was designed to develop consistent superficial dermal to full-skin-thickness SM-induced lesions in weanling pigs, to characterize these lesions using biomechanical measurements, clinical observations, photographs, and histopathology, and to evaluate a temporary wound dressing (TWD, Dermagraft-TC supplied by Advanced Tissue Sciences in La Jolla, CA).

A full-skin-thickness SM burn in this swine model was created over most of the 3 cm diameter lesion with 30 min exposure to 400 μL of undiluted SM, but a 1 hr exposure may be preferable in future studies. The severity of the wound observed on day 2 with the 30-min exposure is considered a deep dermal to full-skin-thickness burn depending on if a periferal or center biopsy sample and in time will become full-skin-thickness. For a partial-skin-thickness burn, the exposure time should be increased to between 5 and 10 min in future studies. The 2-min exposure sites were inconsistent and not uniform within and between animals. Histopathology confirmed that a uniform, consistent partial-skin thickness burn had not been attained with this exposure. Histopathologic data obtained in the pilot study and in study animals predict that a 7-min SM exposure time would provide a consistent partial-skin-thickness burn. Systemic effects were not noted in Phase III, Part C from SM exposure. No differences between the onsite and offsite control sites were observed when evaluated using ultrasound, Chroma Meter, Evaporimeter, or Laser Doppler instruments. These instruments, as well as control sites on the same animal, are recommended for future studies evaluating SM induced lesions.

Overall, pig skin autograft sites healed faster and wounds at 38 days were less severe compared to Dermagraft-TC (DGTC) and no treatment sites. In addition, DGTC sites healed more rapidly than no treatment sites. Autograft and DGTC adherence at graft sites was incomplete for most lesions across all animals evaluated. Damaged tissue was not completely removed by the dermatome method of debridement, and neither graft adhered to the damaged tissue. Microorganisms identified on the lesions are those typically found in the air (Staphylococcus aureus) or from the gastrointestinal tract (Enterococcus, Echericia coli). Since these microorganisms were identified in the last three weeks in one or more lesions per animal,

microorganisms may typically be observed in burns late in the healing process (as wound is drying). Future studies should include identification and quantification of microorganisms at wound sites over the course of the study.

PHASE I

In Phase I, full-skin-thickness SM-induced lesions were produced at six ventral abdominal sites on female, Yorkshire weanling swine (6-20 kg) purchased from Isler Genetics (Prospect, OH). Each lesion was produced using 400 μL of undiluted SM applied cutaneously for 2 hr and had an average size of 1406 mm^2 (an elliptical model was used for all phases). Based on articles describing casualty wounds and treatments during the Iran-Iraq war and on USAMRICD directions for application of therapies, day 2 was chosen to assess, debride, and treat damaged sites.[3,4] Phase I, Part A, assessed debridement and bandaging techniques. Some lesions were debrided using a dermatome set at a 1-mm depth to prepare graft sites in Phase I, Part B, and in Phase II. A partial-skin-thickness autograft was taken from the rump with the dermatome set at a depth of ~0.75 mm. Grafts were attached at lesion sites using surgical staples. All sites were bandaged to provide pressure for graft adherence. In Phase I, Part B, lesions were characterized over a 9-day period using clinical observations, with histology confirmation, and selected clinical chemistry, hematology and urinalysis parameters.

On day 2 clinical observation, minimal exudate was observed at most lesions, with an increased incidence of exudate at anterior sites. This may be related to SM irritation and the animal's ability to reach these lesions with rear feet. Erythema and edema were rated at near maximum, and necrosis of lesions was of moderate or less severity. Erythema heavily skewed results when an overall wound severity (WS) score for each lesion was attempted. Overall site effect was statistically significant for WS, exudate, and edema. Wound size measurements were consistent early, particularly on day 2 evaluations, and variability increased with time. The wounds progressed in size with increasing severity through day nine. There was variability between individuals making clinical observations, and efforts were made to maintain the same evaluator throughout a study. The anterior site wounds appeared less severe than the posterior site wounds, and treatments needed to be rotated through sites to reduce bias. Using a single form for clinical observation criteria for both wound development and wound healing was

impractical, and this led to the creation of two separate evaluation forms. The first evaluation form was for wound development and emphasized wound severity, size, and depth. The second form was for wound healing and emphasized evaluation of the amount of granulation tissue, neovascularization, epithelialization, the presence or absence of infection, wound size, and wound contraction. Clinical evaluations within and between animals were more variable than histopathologic endpoints.

Microscopic evaluation confirmed the development of consistent full-skin-thickness lesions by day 2 greater than the 3-cm diameter application site. Histopathologic assessment of epidermal ulceration, neutrophil infiltration, and necrosis in the epidermis, dermis, basal epithelium, and subcutaneous tissues at day 2 was valuable for grading wound severity and depth. Histologically, the wounds were fully developed and most severe by day 8. These wounds induced a neutrophilia over the seven-day observation period, with localized hemolysis and urinary excretion of the breakdown products of the cutaneous lesion (protein, red blood cells, and hemoglobin).

Clinical chemistry parameters which were outside normal ranges were alanine transaminase (ALT), lactate dehydrogenase (LDH), creatine kinase (CK), and blood urea nitrogen (BUN). ALT was elevated, possibly due to erythrocyte hemolysis in the area of the burn. Evaluation of LDH and CK indicated no significant damage to skeletal or cardiac muscle, except for one animal (elevation of MM isoform of CK related to muscle damage) on day 7, which may have been due to multiple injections and biopsies rather than SM-induced damage. The BB isoform of CK, found in brain, smooth muscle, thyroid, and prostate, showed increases by day 1 to 3 in 4 of 6 animals, and may be associated with daily anesthetization. A slight increase in BUN values was observed by day one, however the increased values were still within the normal range. The BUN:creatinine ratio showed a slight increase, but did not appear to be clinically significant. No significant changes were noted in any of the other clinical chemistry parameters measured.

Hematology identified increases in white blood cell counts, primarily attributed to neutrophilia. An occasional rise in lymphocyte numbers was observed. Increases in neutrophils and lymphocytes were likely the result of stress, SM-induced damage, the wound healing process, and secondary infection of cutaneous lesions. Leukopenia, as reported previously by Gold and Scharf,[39] was not observed in hairless guinea pigs. Trends in decreasing hematocrit,

hemoglobin, red blood cell count, and mean corpuscular hemoglobin concentration were slight and attributed to hemolysis at the SM-lesion and to the multiple blood samples collected. Platelet counts decreased, particularly on day 1 and 3. Multiple venipunctures necessary for blood collection could result in reduced platelets. No significant change was observed in any other hematology parameter measured.

Urinalysis found blood was present in the urine and peaked by day 3, and then declined during the remainder of the 8-day study. This is consistent with the hemolysis noted at the dosed sites. Elevated protein (albumin) levels in the urine occurred by day 3 and remained elevated through the remainder of the observation period. Urine volume increased slightly toward the end of the study. Recovery from anesthesia during wound development on days 0 and 1, and an increased incidence of anesthesia for data collection early in the study, would decrease water intake and thus reduce the amount of urine being eliminated early in the experiment. Nitrites/nitrates in the urine were not detected in 83 percent of the animals on day 0, but were present in all animals by day 7. Nitrites/nitrates are associated with fecal contamination and microbial activity of gram negative bacteria. Leukocytes were not found in any urine sample and urine cultures were not performed. No significant changes were noted in any of the other urinalysis parameters measured.

Thiodiglycol is a major metabolite of SM, and was present in urine samples at the earliest time point tested (6 to 8 hr) with levels ranging from 0.66 to 4.98 μg/mL and a mean of 2.14 μg/mL. Peak levels were observed in half of the animals at this earliest time point of 6 to 8 hr, and between 24 and 48 hr for the remaining animals. The mean level of thiodiglycol by the evening of day 3 had declined to ~50 ng/mL. The mean levels were between 10 and 40 ng/mL for the remainder of the 7 day observation period.

PHASE II

Phase II was performed to evaluate a temporary wound dressing, Dermagraft-TC™ (DGTC), and autograft treatment of full-skin-thickness SM-induced lesions in weanling swine. Overall, autograft sites healed faster and lesions at 38 days were less severe than at DGTC and no-treatment sites. DGTC sites healed more rapidly than no-treatment sites. A comparison of clinical observation data on day 2 between Phase I Part B and Phase II data showed a statistical

difference for the parameters erythema, edema, necrosis and wound size. These differences may be the result of 1) Animal conformation - SM application on deep-chested animals is not as uniform as that on round-chested animals, 2) Dosing site location – Anterior sites are generally smaller and less uniform (observed) than posterior sites due to a flat abdominal surface for SM application and not a sharply angled chest surface, 3) Larger n (n = number of animals) in Phase II – Greater variation in animal size and shapes/conformation, 4) A change in evaluator – The primary evaluator was not available for all observation points over the study time course (Jan 1997 through Aug 1997). This may have contributed to differences in evaluation observations, 5) Changes in SM vials – Over the course of the study, several vials (lots) of SM were used to complete the study. Potency, although within the acceptable range, varied. This indicates that clinical observation evaluations over time are not consistent and should not be considered a primary endpoint for future testing. Autograft and temporary wound dressing adherence at grafts sites was incomplete at most lesion sites across all animals evaluated. Necrosis at the center of most grafts was evident (46 of 48 autograft sites and 25 of 48 DGTC sites) by day 10. Various portions of the grafts were adhered to the dermatomed area by day 7, particularly at the periphery of autografts. Bandages were removed by day 17 and non-adhered autografts had generally sloughed by this time. DGTC remained adhered for longer periods, but were either found in the bandaging material or in the caging when scabs were sloughed. The non-adherence of graft material to the wound bed was due to the following reasons: 1) A single pass with the dermatome (1 mm depth) did not remove the necrotic tissue completely, 2) The graft contact pressure with the wound bed was not great enough to support adherance, 3) Silvadene® cream 1 percent was used as a topical antimicrobial agent and is reported toxic to grafts by Dr. Sheridan of Shriners Burns Institute- Boston Unit, Massachusetts General Hospital, Boston, MA, and 4). Frequent bandage changes (~3 times per week) disturbed the friable neovasuclar beds of the wound. The necrotic tissue in the center of the debrided burn was supported by the histopathology on day 38 for both graft groups (epithelial growth over granulation tissue filled in the dermal defect). The dermatome, set at its thickest setting (1 mm and a single pass performed), did remove necrotic tissue from the periphery of the wound but not from the center.

The scoring system used for adherence, durability, and rejection of the graft during the course of the study appeared inadequate after the graft had either embedded and was viable

(autograft) or sloughed (autograft and DGTC). All 48 sites were evaluated, but there was inability to score the lesions using the categories provided. Future studies will incorporate a revised clinical observation procedure to address this issue.

Wounds often split along the posterior edge. These areas appeared to be healed on observation, but a hematoma often was in areas of incomplete healing in deeper tissue. Histopathology on day 38 indicated that necrosis in the dermal tissue was near maximum at all sites. Granulation/fibrotic tissue was filling in the defect, however it was incomplete by day 38. Since the dermatome did not remove all of the necrotic tissue, areas of incomplete healing are anticipated despite epithelialization of the lesion.

The microorganisms identified in the lesions were those typically found in the air (Staphylococcus aureus) or from the gastrointestinal tract (Enterococcus, Escherichia coli).[5] These microorganisms are believed to be contaminants (wound surface swabs) of the wound and not an infection. Most organisms found are opportunistic and can be pathogenic. Since these microorganisms were identified in the last three weeks in one or more lesions per animal, they typically may be observed in burns as contaminants. Contamination of wounds are generally identified in surface swabs and infections by biopsies taken from the perifery of the wound. The microorganisms observed most frequently were those also reported in human beings.[5] Future studies should include identification and quantification of microorganisms at wound sites over the course of the study.

PHASE III

Part A, Solubility, Stability, and Hydrolysis Testing: Propylene glycol can not be used in SM dosing because it does not mix with SM. Since PEG 400 was not soluble in the gas chromatography (GC) solvent, it was not tested with SM. SM/peanut oil and SM/PEG 200 mixtures at 25, 50 and 75 percent SM levels were stable for more than 8 days when stored at room temperature and more than 35 days when stored in a freezer at approximately -70° C.

Part B, Anesthesia Effects: When taking Laser Doppler readings, three anesthesia regimens resulted in significantly different responses, but none was preferred. The anesthesia regimens behaved similarly within the same day. That is, Laser Doppler readings for each

anesthetic regimen recorded for the four rounds per day generally decreased over the first three rounds and increased slightly for the fourth round. Laser Doppler readings for anesthesia regimens X (repeated injections of Telazol®/Xylazine solution) and W (repeated injections of Ketamine and Xylazine) declined over multiple treatment days, while regimen Y (Telazol®/Xylazine solution as pre-anesthetic to Isoflurane inhalation anesthesia) readings were less consistent in response. No regimen was clearly preferable for taking Laser Doppler readings.

For Evaporimeter readings, anesthesia W resulted in a lower response than with X and Y. There was a decline in response over time within the same day indicating that the Evaporimeter was sensitive to time post-anesthesia. This may be due to dehydration of an animal during anesthesia. The dehydration effect may be compensated for by fluid administration during the anesthesia or taking the Evaporimeter readings within a specified time interval from onset of anesthetic administration. The Evaporimeter response also declined over multiple treatment days with each anesthesia. Regimen X or Y was preferred for taking Evaporimeter readings.

For Chroma Meter readings, anesthesia regimens X and Y resulted in a higher response than W, probably indicating a greater skin blanching effect for anesthesia W. The anesthesia regimens behaved similarly within the same day, with the first readings giving a higher response than subsequent rounds. Response over multiple days was less variable for anesthesia X and W than for Y. Either regimen X or W was suitable, with X preferred due to less skin blanching.

Regimen X is recommended for use in future experiments as it is the most suitable when readings are taken with all three instruments. It is also recommended that Chroma Meter and Evaporimeter readings be taken first, as they are most apt to change over time. Both instruments are time sensitive, however, a pilot study was not conducted to compare the sensitivity between the two instruments. Time constraints (2 hr) established the instrument evaluation order as Chroma Meter, Evaporimeter and Laser Doppler.

Part C, Development of Full-Skin-Thickness and Superficial Dermal Sulfur Mustard-Induced Burns by Varying the Exposure Time

The pilot study selected an exposure time of 30 min for producing a full-skin-thickness lesion and 2 min for a partial-skin-thickness lesion using 400 μL of undiluted sulfur mustard (SM) applied to ventral abdominal sites of swine. Histopathologic endpoints were used as the

criteria for the depth of lesions. Depth of lesions in mm, burn severity score, and necrosis of basal epithelium were the key histopathologic endpoints used to select the exposure times.

Lesions produced by varying exposure time were characterized and described using clinical observations (taken on day 2), histopathologic endpoints (taken on day 2), and non-invasive biomechanical readings (taken on day 0 and day 2). Millipore™ water exposed control sites were usually normal. One Millipore™ water site had a histopathologic grading of "present" for hemorrhage and less than 10 percent of the area involved in basal cell necrosis and necrosis of the epidermis. This is probably an incidental finding due to a scratch or bump from handling, clipping, or moving/lying down in the cage.

The study showed sites exposed for 30 min to 400 µL of undiluted SM were generally erythematous (moderate to severe scoring, and a mean Chroma Meter difference of 8.89 ± SE 0.63), edematous (mean area of 842.91 mm^2 and mean height of 4.3 mm), a mean depth of burn of 1.36 mm ± SE 0.29, and generally the entire dosed area was affected with some extension beyond the dosed site for most day 2 clinical and histopathological endpoints. Necrosis affected the entire epidermis through the basal cell layer and extended into the dermis in at least 86 percent of the sites. Vascular necrosis affected 10 to 50 percent of the lesions at 94 percent of the sites. Trans-epidermal water loss was present with a measured difference of 3.73 ± SE 1.57 between day 0 and day 2. The mean Laser Doppler measurements normalized to offsite controls was reduced (0.96 ± SE 0.08) compared to the control group (1.31 ± SE 0.07). The reduced blood flow resulting from damaged vessels in the affected sites of the 30-min exposure time group was significantly different from that in control animals.

Sites exposed for 2 min to 400 µL of undiluted SM were generally erythematous (pink to deep red, with red being observed predominantly, and a mean Chroma Meter difference of 8.4 ± SE 0.63), with no obvious edema at 48 hr, a mean burn depth of 0.76 mm ± SE 0.29, and generally multifocal areas of damage among normal-appearing areas within the dosed site. Necrosis affected the entire epidermis, with some extension into the dermis. Ninety four percent of the sites had a necrosis of basal cell layer severity score grade of less than 2, indicating that only 10 to 25 percent of the potential area was affected. Vascular necrosis affected less than 10 percent of the potential area at 22 percent of the sites, with the remaining sites graded as normal. Trans-epidermal water loss was present with a measured difference of 3.73 ± SE 1.57 between day 0 and day 2. The mean Laser Doppler measurements normalized to off site controls

was 2.11 ± S.E 0.08. An increase in blood flow to the affected area was observed in the 2-min exposure group when compared to control and 30-min exposure time groups.

There was mixed evidence that the control, partial-skin-thickness and full-skin-thickness groups were statistically significantly different from each other. Histopathologic evaluations and descriptions were key in characterizing the 2-and 30-min exposure time lesions. The severity of the wound observed on day 2 with 30-min exposure was considered deep dermal to full-skin-thickness. Hispathology reported that a full-skin-thickness burn was observed over at most 50 percent of the exposed area in 94 percent of the sites (as determined by vascular necrosis scoring and general pathologist impression). If the entire lesion area is to be full-skin-thickness in all SM exposed sites, then the SM exposure time should be increased. A 1 hr exposure time for a full-skin-thickness burn by day 2 is preferable in future studies. The 2-min exposure time to produce partial-skin-thickness lesions should be increased to between 5 and 10 min in future studies. The 2-min exposure time lesions were inconsistent within and between animals. Histopathology confirmed that a uniform, consistent partial-skin-thickness burn was not attained with the 2-min exposure time. Histopathology data obtained from the pilot study and from this study predict that a 7-min SM exposure time would provide a consistent partial-skin-thickness lesion.

Systemic effects due to SM-exposure were alluded to in Phase I, Part B, and found to be clinically insignificant in earlier experiments conducted under MREF Task 94-33. In Phase III, Part C, no systemic effects of SM-exposure were noted at control sites (C1 and C2) using Ultrasound, Chroma Meter, Laser Doppler, and Evaporimeter readings. Therefore, the use of within animal control sites is recommended for future experiments utilizing this model and instrumentation.

The biomechanical instruments used in this study are non-invasive and are routinely used in dermatology clinics. Their continued use is recommended, particularly for screening treatments and especially for products that pass screens with multiple species. A weanling swine model has been developed that produces SM-induced full- and/or partial-skin-thickness burns for testing efficacy of pretreatment and therapeutic moieties, topical skin protectants, and laser debridement.

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TASK 94-33

In Vivo Evaluation of Temporary Wound Dressings For Adherence, Durability and Autografting on Sulfur Mustard-Induced Lesions in Weanling Swine.

1.0 INTRODUCTION

The U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) is responsible for evaluating treatment regimens for percutaneous bis (2-chloroethyl) sulfide (sulfur mustard; SM) exposure and for developing models to make these assessments. SM, a chemical threat agent, is an alkylating agent and can produce incapacitating vesicant injuries. Chemistry, mechanism of action, and genotoxicity of SM, as well as clinical presentation, toxicodynamics, pathogenesis, and histopathology following SM exposure have been intensely studied and reported.[1-26,33-46] Cutaneous exposure to SM has identified the major target as the rapidly proliferating basal cell in the epidermis. Loss of integrity of the basal cells and anchoring filaments result in the separation of the epidermis from the dermis at the dermal-epidermal junction. The lamina lucida of the basement membrane appears to be the location of separation, resulting in fluid-filled blisters in humans and microblisters in most animal models.[9-14,16-26]

Guinea pigs (both normal and hairless), rabbits, and swine are primary models of choice for dermatological and burn research.[17-27] Hairless guinea pigs have been the species of choice for vesicant research at the United States Army Medical Research Institute of Chemical Defense (USAMRICD).[32-41] The source of hairless guinea pigs was interrupted in 1993 by an outbreak of *Listeria monocytogenes* at the breeding facility of the commercial supplier.[47] Thus, an alternate animal model was required.

The skin of the domestic pig has been reported to have morphologic, pathologic, and functional characteristics comparable to that of the human being.[22-30] These common characteristics include: 1) sparse-hair coat, 2) a thick epidermis with distinct rete pegs and corresponding dermal papillae, 3) a relatively high concentration of elastic fibers in the dermis, 4) hair follicles with similar vascularity, 5) similar collagenous tissue framework and adipose chambers in the subcutis, 6) comparable enzyme patterns in the epidermis with similar apocrine glands, 7) analogous epidermal tissue turnover time and keratinous protein character, and 8) similar composition of the lipid film of the skin surface.[22-30] There are also, however,

differences. The following characteristics of domestic pig skin differ from those of human skin: 1) vascularity of the cutaneous glands and subepidermal plexus is poor, 2) eccrine glands are absent in the common integument, 3) extensive fat is deposited below the subcutis, 4) there is a positive alkaline phosphatase reaction in the basal stratum of the epidermis, 5) a negative or slightly positive reaction for hydrolytic enzymes (particularly alkaline phosphatase) occurs in the capillaries of the subepidermal vascular plexus, 6) strong enzyme reactions occur in active connective tissue cells of the dermis, 7) seasonal shedding of hair exists, and 8) apocrine skin glands are not involved in thermoregulation.[22-30] The weanling pig was selected for the SM burn model because of its similarity in skin to that of human beings, and because the pig exhibits microvesication, or epidermal-dermal separation.[22-30] In addition, pigs are readily available and easily handled, and USAMRICD has used the weanling swine for efficacy testing of topical skin protectants, laser debridement of SM-induced burns, and is developing a historical database.[18,22-26,42]

2.0 OBJECTIVES

This study was designed to develop consistent superficial dermal to full-skin-thickness SM-induced lesions in weanling pigs, to characterize these lesions using biomechanical measurements, clinical observations, photographs, and histopathology, and to evaluate a temporary wound dressing (TWD). The study was conducted in three phases. The first phase was a feasibility study to determine if consistent partial- to full-skin-thickness SM-induced lesions could be produced in a weanling pig. A variety of pretreatments and SM volume-exposure time combinations were tested to determine a regimen that consistently would produce deep partial- or full-skin-thickness SM burns. Two additional parts were added to Phase I. In Part A, technicians were trained in bandaging and debridement techniques. In Part B, six animals were used as control animals for Phase II and to characterize the lesion. The full-skin-thickness lesions produced by SM were evaluated on day 0, 1, 3, and 7, and clinical chemistry and hematology were performed to detect systemic effects. Urine was collected twice daily and analyzed for thiodiglycol. Clinical observations were conducted on day 2, and histopathologic confirmation of SM full-skin-thickness lesions was conducted on day 2 and again ~7 days later. Phase II evaluated the use of a TWD for effectiveness in treating SM-induced dermal lesions. The TWD used in Phase II was Dermatograft-Transitional Covering™ (DGTC), provided by

Advanced Tissue Sciences of Torry, CA. Phase III was to evaluate the effect of anesthetic regimens on wound and biomechanical measurements (laser Doppler, Evaporimeter, Chromo Meter, and ultrasound), to develop and characterize superficial dermal and full-skin- thickness SM-induced wounds, and to determine the feasibility of creating a larger wound (at least 20 percent of the body surface area) using dilutions of SM. This phase was proposed in four parts, with only three parts being accepted. These were: Part A, Solubility, Stability, and Hydrolysis Testing of SM Diluents; Part B, Anesthetic Effects; and Part C, Development of Full-skin-Thickness and Superficial Sulfur-Mustard-Induced Burns by Varying the Exposure Time in Weanling Swine.

3.0 MATERIALS AND METHODS

Female, Yorkshire, weanling swine (6-20 kg) from Isler Genetics (Prospect, OH) were used in a step-wise approach to develop an animal model for deep partial- or full-skin-thickness SM-induced burns. Isler Genetics, Inc. is a specific pathogen free (SPF) facility and an animal source approved by Battelle's Attending Veterinarian. Tables I-3 and 4 in Attachment I identify the animals used in this study. Table 1 presents the number of animals used in the different experimental phases.

Table 1. Animal Usage by Phase

Task 94-33 Phase and Part	Animal Usage
Phase I Development	16
Phase I Part A	6
Phase I Part B	6
Phase II Efficacy Study	31
Phase III Part A	0
Phase III Part B	6
Phase III Part C	25

Animals were quarantined upon receipt for a week, and physical examinations were performed by a staff veterinarian. Each animal was ear tagged with a number composed of the year of birth, litter number, and pig number. Feed was provided twice a day. Animals were fed *ad libitum* initially and then this was changed to the following regimen when prolapses and gastrointestinal bloating occurred. Approximately 0.3 kg per feeding was given initially and this

was increased gradually to approximately 1 kg per feeding for animals maintained for up to eight weeks (Phase II). Animals were weaned from the swine producer's ration to a laboratory swine grower ration manufactured by PMI Feeds, Inc (St. Louis, MO). For animals being anesthetized, feed was removed a minimum of 8 hr prior to anesthesia. Following recovery from anesthesia, feed was gradually increased over several days until a full ration was resumed. Water was supplied from the Battelle West Jefferson water system, and was given *ad libitum*. No contaminants that would affect the results of the study are known to be present in the feed or water.

Once exposed to SM, animals were individually housed in mobile, large animal enclosures (Britz-Heidbrink, Inc., Wheatland, WY). Animals were maintained under fluorescent lighting with a light/dark cycle of 12 hr each per day. Air temperature in swine rooms was maintained between approximately 50 and 80 F. The room temperature was elevated to the upper end of the range at receipt of weanling swine and gradually reduced over time to approximately 65 F. Relative humidity in animal rooms was maintained between approximately 30 and 70 percent.

Animals at Battelle are cared for in accordance with the guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" (National Academy of Sciences, 1996), and/or in the regulations and standards as promulgated by the Agricultural Research Service, United States Department of Agriculture (USDA), pursuant to the Laboratory Animal Welfare Act of August 24, 1966, as amended. On January 31, 1978, Battelle's Columbus Division received full accreditation of its animal care program and facilities from the American Association for Accreditation of Laboratory Animal Care (AAALAC). Battelle's full accreditation status has been renewed after every inspection since the original accreditation. The MREF is a part of the facilities granted full accreditation.

Phase I was designed to develop consistent full-skin-thickness SM-induced lesions in a weanling pig and was divided into three parts. The first part was to develop the model (n=16). The second part, Part A, Bandaging and Debriding Techniques, trained technicians in bandaging and debriding techniques (n=6). In the third part, Part B, Characterization Of Full-Skin-Thickness SM-Induced Burns, full-skin-thickness SM burns were created and half of the six lesions per animal were debrided, and clinical chemistry, hematology, urine and histopathology data were collected for up to 8 days (usually 7 days following debridement on day 2.

3.1 Model Development

3.1.1. Pre-dosing Preparation

Within 24 hr prior to dosing, each animal was weighed. The first 16 swine used in Phase I weighed between 7 and 19 kg. An earlier study indicated clipping of the coarse hair over dose sites frequently left scratches and erythema in the dosing area, which could be observed for at least 24 hr after clipping. The Study Director and technical point of contact (TPOC) at USAMRICD discussed the necessity for hair removal and decided that depilation was necessary. The creation of full-skin-thickness burns using limited quantities of SM without topical pretreatment was considered impractical. Therefore, pretreatments were selected to facilitate hair removal (chemical depilatory), or keratin layer removal (tape stripping, enzyme products), or to assist SM penetration into the epidermis (defatting agent) with minimal irritation. A chemical depilatory compound (Nair® AG, Carter Products, NY, NY) applied to the dosing area for approximately 7 min before rinsing created minimal to no observable irritation the next day. Enzyme products (trypsin or papain) were generally applied the day prior to dosing and removed with a mild soap solution before dosing as described below. A defatting agent (isopropyl alcohol), tape stripping, or abradement (needle, emery paper, or sandpaper) was used immediately prior to placement of the animal in the hood for dosing. Removal of pretreatment products consisted of gentle washing with a 1:20 dilution of Ivory® dish-washing detergent (Proctor and Gamble, Cincinnati, OH) in distilled water. The area was gently patted with warm water-soaked 4x4 gauze sponges (The Kendall Company Hospital Products, Boston, MA), then gently patted with dry gauze sponges or a clean towel to dry. Table 2 summarizes the pretreatments and SM volume and exposure time for each animal and each site.

On the first animal, trypsin was applied to the right dorsum for approximately 2 hr on the day of dosing. Tape stripping was accomplished on the left dorsum prior to dosing. Trypsin was removed, as described above, before dosing with SM. The ventral abdomen was tried on the second animal since the first animal had only erythema. The second animal had trypsin and papain (Meijer, Grand Rapids, MI) applied to the right and left side of the ventral abdomen, respectively, and then was covered with a stockinette (Health Products Division, Alba-

Waldensian, Inc., Rockwood, TN) overnight; washing before dosing was accomplished as described above. Histopathologic evaluation of lesions on the first animal indicated the lesions were superficial burns to superficial dermal burns (varying depths of dermal necrosis). Discussions with the COR and TPOC moved the dosing site from the dorsum to the ventral abdomen. The skin on the ventral abdomen is thinner than that on the dorsum. Animals two through eleven had trypsin (Trypzyme-V®, Veterinary Products Laboratory, Phoenix, AZ) applied to the designated dosing area (ventral abdomen right and left side of the teat line) and covered with a stockinette overnight. Animals 12 through 16 had only Nair® applied to the ventral abdominal dosing area the day before dosing.

Table 2. Pretreatment and Dosing Summary for Sixteen Animals Used to Develop Sulfur Mustard-Induced Chemical Burns

Animal Number	Animal ID	HD Dose[1] (µL-hr)	Right Side Pretreatments									Left Side Pretreatments								
			Site	Nair[2]	Trypsin[3]	Papain[4]	Tape Strip	Sand paper	Emery Paper	Needle	Defat	Site	Nair[2]	Trypsin[3]	Papain[4]	Tape Strip	Sand paper	Emory Paper	Needle	Defat
		200-2 (A&B)	B	x	x							A	x			x				
		200-1.5(C&D)	D	x	x							C	x			x				
1	96-13-9[5]	200-1 (E&F)	F	x	x							E	x			x				
			A	x	x					x		B	x		x				x	
			C	x	x			x				D	x		x		x			
2	96-13-7	500-2	E	x	x							F	x		x					
			A	x	x					x		B	x	x					x	x
			C	x	x			x				D	x	x			x			x
3 & 4	96-21-7 96-21-10	300-2	E	x	x				x			F	x	x				x		x
		300-2 (A&D)	A	x	x							B	x	x		x				
		500-2 (B&C)	C	x	x							D	x	x		x				
5	96-34-5	600-2 (E&F)	E	x	x							F	x	x		x				
			A	x	x							B	x	x						
			C	x	x							D	x	x						
6	96-34-4	300-2	E	x	x							F	x	x						
			A	x	x							B	x	x						
	96-1-4 96-1-3 96-3-9		C	x	x							D	x	x						
7-10	96-2-10	400-2	E	x	x							F	x	x						

Table 2. Pretreatment and Dosing Summary for First Sixteen Animals Used to Develop Sulfur Mustard-Induced Chemical Burns (Continued)

Animal Number	Animal ID	HD Dose[1] (µL-hr)	Right Side Pretreatments									Left Side Pretreatments								
			Site	Nair[2]	Trypsin[3]	Papain[4]	Tape Strip	Sand paper	Emery Paper	Needle	Defat	Site	Nair[2]	Trypsin[3]	Papain[4]	Tape Strip	Sand paper	Emory Paper	Needle	Defat
			A	x	x							B	x							
			C	x	x							D	x							
11	96-5-8	400-2	E	x	x							F	x							
	96-10-12 96-10-11 96-11-8 96-11-11		A	x								B	x							
			C	x								D	x							
12-15		400-2	E	x								F	x							
			A	x								B	x							
			C	x								D	x							
16	96-18-8	400-2	E	x								F	x							

[1] All SM doses were applied for 2 hours, except for animal 96-13-9. On this animal, sites A & B were dosed for 2 hrs, C & D were dosed for 1.5 hours, and E & F were dosed for 1 hour.

[2] Nair was generally applied for 7 minutes. Occasionally, two applications were necessary for complete hair removal.

[3] Trypsin was applied overnight, except for animal 96-13-9. Trypsin was applied for 2 hours on this animal.

[4] Papain was applied overnight.

[5] Animal 96-13-19 was dosed on the dosum. All other animals were dosed on the ventral abdomen.

3.1.2. Dosing Day Preparation (Day 0)

Enzyme pretreatments were removed on the day of dosing before drawing the dosing grid, except for the first animal dosed. Animals were anesthetized for pretreatments, SM exposure, wound excision and/or biopsy, and treatment application (autograft and DGTC application). Atropine sulfate (AMVET Scientific Products, Yaphank, NY) was administered intramuscularly (IM) at 0.04 mg/kg body weight to control excessive salivation. Animals were anesthetized with a combination of xylazine hydrochloride (Ben Venue Laboratories, Inc., Bedford, OH) and Telazol® (Fort Dodge Laboratories, Inc, Fort Dodge, IA). Five mL of xylazine hydrochloride solution (100 mg/mL) were used to reconstitute Telazol® (250 mg tiletamine and 250 mg zolazepam) and the mixture dosed at approximately 0.044 mL/kg BW IM. Each animal had an endotracheal tube placed for Isoflurane (Abbott Laboratories, North Chicago, IL) inhalation anesthesia. Anesthesia was initiated using a concentration of 2.5 to 3 percent Isoflurane in oxygen at an initial flow rate of 2 L per min using an Anesco anesthetic machine (Anesco, Inc, Georgetown, KY) or a Matrix anesthetic machine (Matrix Medical Inc., Orchard Park, NY). Generally, Isoflurane concentration was reduced to approximately 1-1.5 percent and oxygen flow rates reduced to a range of 800 mL to 1 L/min to maintain anesthesia. Pretreatments were accomplished and/or dosing templates were applied once the animal was anesthetized. Following SM exposures, the concentration of Isoflurane was reduced over time until the animal was on 100 percent oxygen, and then gradually changed to room air.

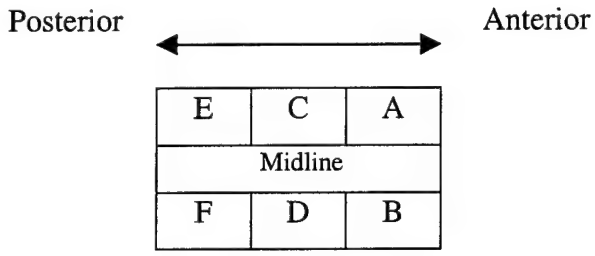
3.1.3. Pretreatment Techniques to Facilitate SM Penetration of the Epidermis

The keratin layer of the skin was stripped away to facilitate SM penetration into the epidermis in order to produce a deep partial- to full-skin-thickness burn. Techniques used were tape stripping, abrading, or enzyme treatment. For tape stripping, a section of duct tape (3M, St. Paul, MN) was used to form a loop, adhesive side out, encircling the applicator's fingers, and was applied once in a rolling motion over the designated dose site. For abrading dose sites, a 25 ga hypodermic needle (Becton Dickinson Co., Rutherford, NJ), a 3M scouring pad (3M), sandpaper (#100, Sears, Roebuck and Co., Chicago, IL), or emery paper (3M) was used once over the designated dose site. Enzyme therapy was with trypsin or papain, and was used in combination with some of the abrading techniques. Defatting of the skin was performed just

before application of dosing templates or abrading, using isopropyl alcohol-soaked gauze dabbed on the area to be dosed. As the model was developed, the decision was made to minimize the preparations before dosing. Pretreatment techniques, except for hair removal, were eliminated.

3.1.4. Application of the Dosing Grid and Templates

A six-site dosing grid (3 sites per side) was drawn on the dorsum (first animal only) or ventral abdomen of each animal, as shown below.



Each grid within the template measured approximately 5 cm by 5 cm. The SM application site within the grid measured a 3 cm diameter circle. Discussions with Dr. Gary Gentzkow, Dr. Lynn Allen, and Dr. Lee Landeen of Advanced Tissue Sciences (La Jolla, CA), and Dr. James Blank and Dr. Frances Reid of Battelle (Columbus, OH) stated that lesions less than 2.5 cm in diameter would heal without requiring a graft. An approximately 0.5-1 cm space separated each dosing square. For dorsal dose sites, with the pig in ventral recumbency, the upper edge of the dosing grid was drawn parallel to and approximately 1.5 cm lateral to the dorsal spinal processes on both sides of the animal. For ventral dose sites, with the animal in dorsal recumbency, the dosing grid was drawn approximately 1.5 cm lateral to and parallel to the teat line on both sides of the animal. Dosing templates were applied to each site prior to placing the animal in a sling within a chemical fume hood. The animal was secured in the sling and allowed to stabilize for up to 20 minutes prior to dosing.

Each dosing template was constructed of a 5 by 5 cm square of duct tape applied to double-sided carpet tape of the same size. A 3-cm diameter circle was cut through the tape assembly, and between the two tapes a Whatman No. 2, microfiber glass filter paper (Whatman, Hillsboro, OR) was placed to cover the 3-cm diameter hole. A 3-cm diameter O-ring (Hewlett Packard, Wilmington, DE) was glued to the outer surface of the tape assembly and surrounded the 3-cm diameter hole.

3.1.5. SM Dosing

SM was provided by USAMRICD. Purity, appropriate identification and storage condition information was provided by USAMRICD. SM samples were taken and evaluated by the MREF chemistry section to assure concentration. Appendix C of Letter Report 1 in Attachment B contains chemistry analysis data. Samples were analyzed in triplicate by gas chromatography using a flame ionization detector (FID). Values ranged from 91.8 to 99.5 percent of expected.

SM exposure times (1, 1.5 and 2 hr) and dosing volumes (200, 300, 400, 500 and 600 μL) were varied to determine conditions for producing deep partial- to full-skin-thickness SM burns. SM was applied to the 3-cm diameter microfiber filter within the dosing template using either a 250 or 500-μL glass Hamilton syringe (Hamilton Company, Reno, NV) with a blunt 18 ga needle. After application of SM, a size 3 teflon disc (Thomas Scientific Company, Swedesboro, NJ) was placed over the dosing site and a rubber stopper inserted in the dosing well to occlude the dose site, minimize SM evaporation, and achieve a uniform SM application. After several animals were dosed, the rubber stoppers were secured on each side using a rubber tile float taped in place using Vetrap® (3M).

3.1.6. Decontamination

After exposures, the first three animals were decontaminated by gently patting each dosed site with a 2 by 3 cm absorbent swab attached to a tongue depressor to remove excess SM. Absorbent swabs soaked with 1.5 mL of a 0.5 percent sodium hypochlorite solution were then applied to each dose site for 10 sec. Next, 1.5 mL of water was dabbed on each site for 10 sec using 2 by 3-cm absorbent swabs, and this was repeated a second time. After dose site decontamination, a 0.5 percent sodium hypochlorite-soaked sports towel (Musslinn Sports Towel, Johnson and Johnson Co., New Brunswick, NJ) was placed over the entire dose grid and covered with a plastic-backed absorbent paper for 4 min. This was followed by 3 water rinses using water-soaked towels applied for 2 min. The dosed sites were allowed to air dry for approximately 15 min prior to placing an approximately 2-L plastic bag over the entire dosed area, and a relatively air-tight seal formed using double-sided carpet tape cut in lengths to secure the bag to the area. A MINICAMS™ (CSM Company, Birmingham, AL) was used to sample the

air within this plastic bag after allowing it to equilibrate for approximately 15 min. The decontamination procedure was altered several times as reported in Appendix M study file notes of May 7, 1996 to attain acceptably low SM levels (≤ 0.5 TWA) before removing the animal from the hood. The first two animals were maintained in the hood for approximately 8 hours before removing them. A third animal had bleach applied to decontaminate the dose sites. Several MINICAMS™ analyses and housing the animal in the fume hood overnight were required to lower the apparent SM level below 0.5 TWA within the sampling bag. The decontamination procedure was altered since chlorine was believed to interfere with the MINICAMS™ readings. Readings were lower when only water was used. The following procedure summary was used until Phase III (see Phase III Materials and Methods, SM Dosing and Decontamination section). Dry gauze sponge was gently pressed against each dose site for 30 sec to remove excess SM. A water-soaked gauze sponge was then gently pressed to each dose site for 30 sec. A water-soaked sports towel was placed over each side of the animal and a plastic-backed absorbent paper sheet was placed over the sports towel and held in place for 1 min. This step was repeated and then the area allowed to air dry for approximately 15 min before application of the plastic bag tent to monitor for SM. The animal was allowed to recover from anesthesia, removed from the sling, extubated, and kept in the chemical fume hood overnight in a modified animal transport crate. The next morning, the animal was anesthetized with the Telazol®/xylazine combination and decontamination evaluated using a MINICAMS™. Once proof of decontamination was obtained, the animal was removed from the fume hood and returned to its pen. The proof of decontamination procedure described in the May 7, 1996 study file notes (Attachment M) was not used.

3.1.7 Euthanasia

Biopsies were taken on different study days for the first 16 animals. Table E-1 of the Phase I Statistics Report in Attachment D reports the biopsy day under the study day column for each animal. Euthanasia was performed on the last day of observations and biopsies. Animals that required histopathology evaluation of the lesions were anesthetized initially, then euthanatized on day of last biopsy using a concentrated solution of pentobarbital sodium, generally Buthanasia® D (Schering Plough Animal Health Corporation, Kenilworth, NJ).

Approximately 1 mL for every 10 pounds was administered intravenously per animal. The animal was pronounced deceased when the heart beat could no longer be auscultated or palpated.

3.2 Part A, Bandaging and Debridement Techniques

Six animals were added to Phase I, Part A, to train technicians to dermatome, debride, and bandage lesions. These animals were prepared and dosed as described previously. In consultation with the USAMRICD Scientific Technical Objective (STO) representative and the Contracting Officer's Representative (COR), several methods of debridement and bandaging were taught using these animals. Surgical excision, surgical removal of epidermis only, and the use of the dermatome (approximately 1-mm skin thickness) for debriding wounds were methods practiced. Application of an autograft and/or Dermagraft-TC™ (Advanced Tissue Sciences, La Jolla, CA) with several bandaging techniques were performed. Day 2 was chosen to debride and graft damaged sites based on articles describing wounds and treatments from the Iran-Iraq war and on USAMRICD directions for development of therapies.[3,4] A summary of the debridement and bandaging techniques practiced on each animal is presented in Summary Report 2 in Attachment C.

3.3 Part B, Characterization Of Full-Skin-Thickness SM-Induced Burns

Earlier testing had shown that multiple biopsies of the lesion altered lesions and introduced new variables with each biopsy. This complicated clinical evaluations of the wounds. In consultation with the STO representative and the COR, the decision was made to add six animals to Phase I, Part B, to serve as the control group for Phase II animals. Physiologic endpoints were measured over a 7-day period after the day 2 debridement. These endpoints were evaluated to determine if there was a systemic effect from dermal application of SM in these animals. Urine was collected and analyzed twice a day for thiodiglycol and limited urinalysis (Bayer Multistix 10 SG, Bayer Corporation, Diagnostics Division, Elkhart, IN) through the 8-day study. Hematology (red blood cell and white blood cell differential counts), and serum chemistry data were collected and measured once a day on days 0, 1, 3, and 7/8 of the study. Histopathologic endpoints were evaluated on day 2 biopsies and day 7 or 8 (one animal was evaluated at day 8), with day 0 being the SM exposure day. Biopsy samples on study day 2 were

taken from the center and a periferal site (6 o'clock position with the clock face oriented such that 12 o'clock was ventral and 6 o'clock dorsal). A full-skin-thickness lesion excision was conducted on study day 7.

Protocol 108, pages 54 to 62, describes the study design for the 6 animals used in Phase I Part B. In summary, all 6 sites per animal were exposed for 2 hr to 400 µL SM as described in Summary Report 1 of Attachment C. On day 2, 3 sites were debrided with a single pass of the dermatome set at ~1 mm and 3 sites were untreated. An example of debrided sites is presented in Figure 1 on page 59 of Protocol 108.

These animals were exposed to SM (as described in Summary Report 1 of Attachment C) and after removal from the chemical fume hood, were individually housed in nursery enclosures (Palco, Inc., Belleplaine, IA) modified for urine collection. Each unit consisted of two side-by-side pens, with each pen constructed with a separate urine collection tray. Urine was collected every 12 hr for approximately 7 days. Animals were moved from the dirty pen of the housing unit to the clean pen every 12 hr. A sterilized glass pyrex dish was used to collect the urine over the 12-hr period. Summary Report 3 in Attachment C describes the techniques used for each animal of Part B, Phase I. A 10-mL aliquot from each urine sample was sent to USAMRICD for analysis of thiodiglycol. Thiodiglycol is a hydrolysis product of SM and can be used to test systemic absorption of SM from percutaneous exposure.[44-47] Urine volume was also recorded every 12 hr.

3.4 Analyses

3.4.1 Model Development

Due to the exploratory nature of these experiments, the statistical analyses focused on summarizing day 2 results for the pretreatment, SM dose volume and exposure time combinations. From these results, a procedure was selected that produced consistent full-skin-thickness SM burns for use in Phase II of this task. Summary Report 1 in Attachment C describes this procedure in detail. The last five animals (96-10-12, 96-10-11, 96-11-8, 96-11-11, and 96-18-8) were pretreated with Nair® only and dosed with 400 µL SM for 2 hr at all six sites. In addition, one other animal (96-5-8) received this combination on the left side (sites B, D, and F).

Incidence determinations were conducted on the histopathology endpoints. Descriptive statistics were conducted on clinical observations. Wound size (WS) measurements for Phases I, II and III were calculated assuming an elliptical wound as follows:

$WS = \pi * L/2 * W/2$
where: WS is wound size,
π is the mathematical constant 3.14159265,
L is the length of the lesion, and
W is the width of the lesion

Analysis of variance (ANOVA) models were fitted to the Wound Severity Score (WSS) and Wound Size (WS) data collected on study day 2 to assess the animal-to-animal variability and to determine whether there were significant differences among sites. Appropriate contrasts were used to assess whether there were differences between anterior and posterior sites. The ANOVA model took the following form:

$Y_{ij} = +\mu+\alpha_i+\gamma_j+\varepsilon_{ij}$
where Y_{ij} is the observed WSS or WS for site i on animal j,
μ is the average WSS or WS,
α_i is a fixed site effect,
γ_j is a random animal effect, and ε_{ij} is a random error term.

The Statistical Analysis System (SAS ver. 6.12, Cary, NC) GLM procedure was used to fit the ANOVA models.

3.4.2 Part A, Bandaging and Debridement Techniques

Statistical analyses were not conducted on these data.

3.4.3. Part B, Characterization of Full-Skin-Thickness SM-Induced Lesions

Six animals were used to evaluate specific physiological, histopathological, and clinical observation endpoints up to an 11 day period. Blood cell counts, and serum chemistry data were collected on study days 0, 1, 3, and 7. Urine volume was measured twice a day for 7 days. Histopathologic endpoints were evaluated on day 2 and on days 7 (2 animals), 8 (3 animals), or 9

(1 animal). Clinical observations were made on day 2. Statistical analysis techniques and methods used for each parameter are presented in the Statistics Reports of April 16, 1998 and July 16, 1998 in Attachment D.

4.0 RESULTS

The statistical analysis results of wound development are presented in Appendix D Letter Report dated November 18, 1997 in Attachment B. Histopathologic statistical evaluation results are presented in Addendum A of the Letter Report dated November 18, 1997 in Attachment B. Histopathologic and clinical observation data summaries for each animal are presented in Appendix E of the same of Letter Report in Attachment B. The statistical analyses were performed on data from the last five animals, whose burns were full-skin-thickness and produced using the techniques selected. The final procedure for lesion development in conjunction with the details is described in Summary Report 1 of Attachment C.

4.1 Model Development

4.1.1 Histopathology Summary

Table 3 under the histopathology section in the Results section of the Letter Report dated November 18, 1997 in Attachment B presents the incidence of histopathology endpoints on sites pretreated only with Nair® and dosed with 400 µL SM for 2 hr. Microblisters and infection were pathologic endpoints not seen in these animals. Dermal hemorrhage was observed infrequently. The following conditions were observed in at least half of the 30 sites on study day 2: epidermal necrosis (93.3 percent), follicular necrosis (60.0 percent), dermal neutrophil infiltration (73.3 percent), subcutaneous hemorrhage (50.0 percent), subcutaneous edema (76.7 percent), and subcutaneous neutrophil infiltration (73.3 percent). Although fewer sites were examined on later study days, high incidences of dermal neutrophil infiltration and subcutaneous neutrophil infiltration were observed through day 23. Epidermal necrosis and follicular necrosis continued to be evident through study day 6, but not later, and subcutaneous hemorrhage and subcutaneous edema were not observed after study day 3, with the exception that subcutaneous hemorrhage

was observed at two sites on day 23. These two hemorrhage sites occurred after the wounds appeared to have healed. Two conditions were observed with high incidence on the middle study days: dermal necrosis on days 3 to 9 and subcutaneous necrosis on days 3 to 16. Incidence of dermal edema and neovascularization peaked on study day 9, with each present in 3 of the 6 sites examined on that day. The remaining histopathologic indicators were more often present on later study days. Granulation, re-epithelialization, and sloughing were observed mostly on days 9 to 23. Epidermal ulceration was observed on day 2 in 1 of 30 sites, on day 9 in 4 of 6 sites, on day 16 in 2 of 13 sites, and day 23 in 6 of 6 sites . Epidermal neutrophil infiltration was also observed on day 2 in 1 of 30 sites, day 6 in 2 of 3 sites, day 9 in 4 of 6 sites, and day 23 in 5 of 6 sites.

A more complete summary of histopathologic evaluations for wound development is presented in Tables A-1 through A-4 of Addendum A of the Statistics Report in the Letter Report dated November 18, 1997 in Attachment B. These tables present incidence of histopathologic endpoints on study day two and later for all pretreatment and SM dose combinations where two or more sites were treated.

4.1.2 Clinical Observations Summary

Descriptive statistics of clinical observations on the same animals are presented in Table 4 of the Statistics Report (Appendix D of Letter Report dated November 18, 1997 in Attachment B). Exudate, erythema, edema, and necrosis were evaluated on days 2, 3, and 6. A clinical wound severity score (WSS) was calculated as the sum of the pathology. Erythema, necrosis, and edema were present at all sites on study day 2, except for one site on animal 96-10-11 where necrosis was not present. Erythema scores tended to be most severe, comprising approximately one-half of the WSS. Exudate was observed infrequently, except on animal 96-18-8 where it was observed at 4 of 6 sites. When observed, exudate was mild. Figure 1 of the Statistics Report (Appendix D of Letter Report dated November 18, 1997 in Attachment B) presents the mean WSS for each animal, overlaid on the observed values for each site. From this figure, it is apparent that the WSS varied considerably, both between sites on an animal and between animals.

Wound size (WS) was measured at each site on each study day. WS was calculated as

described in Task 94-33, Phase I Model Development, 3.4 Analyses section. WS data are presented in Figures 2 and 3 of the Statistics Report (Appendix D of Letter Report dated November 18, 1997 in Attachment B). Figure 2 illustrates the mean WS for each animal on study day 2, overlaid on the observed values for each site. Figure 3 displays the mean WS plotted against time for animal 96-18-8, overlaid on the observed values for each site. On study day 2, WS appears to be fairly consistent between sites on an animal and between animals. For animal 96-18-8, WS is greatest on study day 9, and variability appears to increase with time. However, WS measurements on day 30 appear to be inconsistent with measurements on days 23 and 37. This is primarily attributed to differences in the judgement of individuals taking the measurements, rather than rapid changes in the wounds during this period or to a difference in the number of sites evaluated (3 sites on day 30 versus 6 sites on days 23 and 27).

To evaluate wound healing, indications of exudate, inflammation, granulation, contraction, infection, vascularization, and epithelialization were recorded on study days 9, 16, 23, 30, and 37 for animal 96-18-8. Figure 4 in the Statistical Report (Appendix D of the Letter Report dated November 18, 1997 in Attachment B) presents the mean score for each wound healing parameter, averaged over the 6 sites on animal 96-18-8, plotted against time. The wound healing scores tended to decrease over time, with the exception that inflammation increased through study day 23 and was, in general, not observed thereafter. In addition, granulation scores were higher on day 30 than at earlier times. Granulation was not scored on study day 37 as the wounds appeared to be completely healed.

No significant effects were detected in the analysis of WS. There was significant animal-to-animal variability in WSS ($p<0.001$, $\sigma_{animal}=1.6$). The overall site effect for WSS was not statistically significant ($p=0.22$), when each site was evaluated individually in the ANOVA model. A more specific test comparing the averages for sites A and B to the averages for sites E and F determined that WSS averages were significantly greater on posterior sites than anterior sites ($p=0.03$, estimated difference in means [$\pm$ SE] = 3.1 [$\pm$ 1.3]). The anterior site versus posterior site test is more sensitive than the overall ANOVA test in detecting the difference. These results are not contradictory. In some cases, noise in the data may cloud an effect in the overall test, but a specific effect of interest may be detected by an appropriate hypothesis test.

4.2 Part A, Bandaging and Debridement Techniques

A single pass over the lesion with the dermatome set at the deepest setting of 0.1 mm was the debridement technique chosen to excise the damaged area for Phase II. Table 1 in Attachment C indicates the bandaging technique chosen for grafted wounds.

4.3 Part B, Characterization of Full-Skin-Thickness SM-Induced Burns

Descriptive and/or summary statistics are presented for histopathology, clinical observations, serum chemistry, hematology, and urinalysis of Phase I, Part B, animals in the Statistical Reports (April 16, and July 16, 1998) in Attachment D. The models and description of analytical methods are described in these reports. Unless otherwise noted, no statistical evaluations of values or their trends over time were conducted. Visual inspection of the data indicated possible trends.

4.3.1 Histopathology

Eighteen of the 36 sites were evaluated for each histopathologic endpoint. Sites for which evaluations were made, were given a score and the percent incidence determined. Debridement usually removed the epidermal layer of skin, except at the periphery. Table 3 summarizes the mean score and percent incidence of the histopathologic endpoints. Necrosis was present in all biopsy samples taken from the centers of the exposed sites on day 2 and day 7 regardless of debriding, and was present in all but one peripheral sample on day 2 (a debrided site). The mean grade for depth of necrosis was near maximum (4) at 3.9 for both debrided and non-debrided center sites on day 2. Depth of necrosis was graded at the maximum score of 4 for all center sites on day 7. The mean grade for depth of necrosis on day 2 was lower (less severe) for peripheral sites than center sites, 3.6 for debrided and 3.8 for non-debrided. Necrosis of the basal epithelium was present in non-debrided center and peripheral samples, and in debrided peripheral samples only. The maximum grade of 4 was observed in all these samples on day 2, regardless of debriding or sample location. At day 7, necrosis of the basal epithelium was still present at the maximum grade (4) in all evaluated sites. Ulceration was present in one peripheral

debrided site on day 2, and could not be evaluated in the 18 debrided central samples, but was present in all samples on day 7. There was no sign of tissue granulation in day 2 samples, but on day 7 tissue granulation was present in all central samples. The mean tissue granulation grade on day 7 was 2.8 for debrided sites and 2.4 for sites not debrided. No re-epithelialization occurred by day 7.

Table 3. Mean and Percent Incidence of Histopathologic Wound Development and Wound Healing Endpoints for the Six Animals Tested in Phase I, Part B

	Histopathology Endpoint	Debride Site	Center						Peripheral		
			Day 2			Day 7			Day 2		
			N[a]	%* Incidence	Mean*	N[a]	%* Incidence	Mean*	N[a]	%* Incidence	Mean*
Wound Development	Depth of Necrosis	Yes	18	100	3.9	18	100	4.0	18	94	3.6
		No	18	100	3.9	18	100	4.0	18	100	3.8
	Necrosis of Basal Epithelium	Yes	0	NA	NA	9	100	4.0	4	100	4.0
		No	18	100	4.0	16	100	4.0	18	100	4.0
	Ulceration	Yes	0	NA	NA	18	100	NA	4	25	NA
		No	18	0	NA	18	100	NA	18	0	NA
Wound Healing	Granulation Tissue Response	Yes	18	0	NA	18	100	2.8	18	0	NA
		No	18	0	NA	18	100	2.4	18	0	NA
	Reepithelialization	Yes	18	0	NA	18	0	NA	15	0	NA
		No	0	NA	NA	18	0	NA	0	NA	NA

N[a]= Eighteen sites observed for each debrided and non-debrided group were observed for each histopathologic endpoint in biopsy samples.

*Means were calculated only for sites when the histopathologic endpoint could be evaluated.

Samples were taken from the center and periphery of each site on study day 2 and only from the center of each site on study day 7. "N" represents the number of these sites that were evaluated for that specific histopathologic endpoint within the selected groupings (study day, center or periphery, and debride or non-debride).

4.3.2 Clinical Observations

Descriptive statistics for clinical observations are presented in Table 2 of the July 16, 1998 revised Statistical Report in Attachment D. Exudate, erythema, edema, necrosis, and eschar were evaluated on day 2. Eschar was excluded from the above table since none was observed. WS was measured for each site.

Erythema, necrosis, and edema were present in all sites on study day 2. Erythema and edema scores tended to be the most severe with average scores of 4.68 and 4.47, respectively, while necrosis was more moderate with an average score of 1.23 on day 2. Exudate was observed infrequently, except on animal 97-63-10, where it was observed at 5 of 6 sites. When observed, exudate was mild, except in the anterior sites of animal 97-63-10 where the values were 3 and 2.25. Average WS was 1,565 mm^2. The mean values of exudate, erythema, edema, necrosis, and WS for each animal, overlaid on the observed values for each site are presented in Figures 1-5, respectively, of Appendix D in the Statistical Report dated July 16, 1998 in Attachment D.

Analysis of variance (ANOVA) models were fit to the exudate, erythema, edema, necrosis, and WS data to assess the animal-to-animal variability and to determine if there was a significant difference among sites. A brief description and results are reported in Table 3 of the revised Statistical Report dated July 16, 1998 in Attachment D. The overall site effect was statistically significant for WS, exudate, and edema. WS scores were significantly greater on posterior sites than anterior sites as were edema scores.

Exudate scores were significantly smaller on posterior sites than anterior sites. Animal-to-animal variability was significant for exudate, erythema, edema, and WS. No significant effects were detected in the analysis of necrosis.

4.3.3 Serum Chemistry

Descriptive statistics for serum chemistry values for the pre-study physical through day 7 are presented in Table 4 of the July 16, 1998 revised Statistical Report in Attachment D. The mean, minimum, and maximum values per day are also presented in Figures 6 through 24 of the

July 16, 1998 revised Statistical Report in Attachment D. Additional descriptive statistics are reported in the April 16, 1998 Statistics Report in Attachment D (page D-108).

Figure 8 in the July 16, 1998 revised Statistical Report in Attachment D shows that alkaline phosphatase declines from a mean of 450.5 U/L during the physical to a mean of 343.4 U/L on the day of dosing. It continues to decline after dosing to a mean of 252.5 U/L on day 7. Figure 11 in the same report shows mean blood urea nitrogen levels rising from 10.0 mg/dL on day 0 to approximately 16 mg/dL on days 1, 3, and 7. The ratio of blood urea nitrogen to creatinine profile (Figure 23 of the same report) is similar to the blood urea nitrogen profile as creatinine levels did not change greatly through most of the study. However, as seen in Figure 15 of the same report, creatinine levels are somewhat higher on day 1 and day 3.

4.3.4 Hematology

Descriptive statistics for hematolgy data for the pre-study physical through day 7 are presented in Table 5 of the July 16, 1998 revised Statistical Report in Attachment D. The mean, minimum, and maximum values per day are also presented in Figures 25 through 37 of the July 16, 1998 revised Statistical Report in Attachment D. Additional descriptive statistics are reported in the April 16, 1998 Statistics Report in Attachment D (page D-108). The notable hematologic trends observed are as follows: between day 0 and day 7, mean basophil counts (Figure 25) rise from 0.07 x $10^3/\mu L$ to 0.21 x $10^3/\mu L$. Mean corpuscular hemoglobin concentration (Figure 31) decreases over the course of the study from a mean of 33.3 g/dL on day 0 to 32.3 g/dL on day 7. Mean monocyte levels (Figure 33) rise from 1.3 x $10^3/\mu L$ to 2.5 x $10^3/\mu L$ between day 0 and day 7. Mean neutrophil (Figure 34) values rise from 5.284 x $10^3/\mu L$ on day 0 to 8.32 x $10^3/\mu L$ on day 7. White blood cell counts (Figure 37) rise from a mean of 11.09 x $10^3/\mu L$ on day 0 to a mean of 17.08 x $10^3/\mu L$ on day 7. Mean neutrophil and white blood cell counts were similar on days 3 and 7, however, greater variability was present on day 3.

4.3.5 Urinalysis

Descriptive statistics for urine volume and thiodiglycol concentrations for the morning (AM) of day 0 through the evening (PM) of day 7 are presented in Table 6 of the July 16, 1998 revised Statistical Report in Attachment D. The mean, minimum, and maximum values are also presented in Figures 38 and 39 of the July 16, 1998 revised Statistical Report in Attachment D. The distributions of the results from the reagent strips for urinalysis are presented in Table 7 of the same report. Additional descriptive statistics are reported in the April 16, 1998 Statistics Report in Attachment D (page D-108).

Urine volume increased slightly toward the end of the study. As shown in Table 7 of the July 16, 1998 revised Statistical Report in Attachment D, glucose was detected in the urine of only two animals. On day 1 (PM), 100 mg/dL of glucose was detected in one animal and on day 1 (PM) and day 2 (AM), 1000 mg/dL and 2000 mg/dL respectively were detected in the other. Small amounts of bilirubin were detected in one animal on day 5 (AM and PM). Trace amounts of ketone (5 mg/dL) were detected in one animal on day 3 (AM) and again in another animal on day 7 (AM). Blood levels in urine rose through day 3 (PM), where 83 percent of animals had large amounts of blood detected and the remaining 17 percent had moderate amounts of blood detected. Blood in the urine began to decline toward the end of the study after day 3 (PM). By day 3 (AM), 67 percent of animals had 30 mg/dL protein in the urine, while the remaining 33 percent all showed trace amounts of protein. Protein levels remained elevated at the end of the study. The test for nitrite was negative for 83 percent of animals at day 0 (AM), but was positive for all animals by day 7 (PM). Leukocytes were not detected in urine throughout the study. The pH of the urine samples was highly variable on a daily basis, but generally show a decline in the first 24 to 48 hr followed by a gradual return to baseline. Specific gravity readings were also highly variable, but appear to increase following dosing.

Urine samples were collected and sent to USAMRICD for analyses of thiodiglycol. Results were presented at the 1998 Bioscience Review in Baltimore, MD. The urine volume and thiodiglycol concentration results are presented in Table 3 of the revised Statistical Reports dated April 16, 1998 and Table 6 of the July 16, 1998 (provides additional analyses) and in figures A-36 through A-38 and B-36 through B-38 of the April 16, 1998 revised Statistical Report in Attachment D. Figure 1 presents the total thiodiglycol for each animal. Thiodiglycol

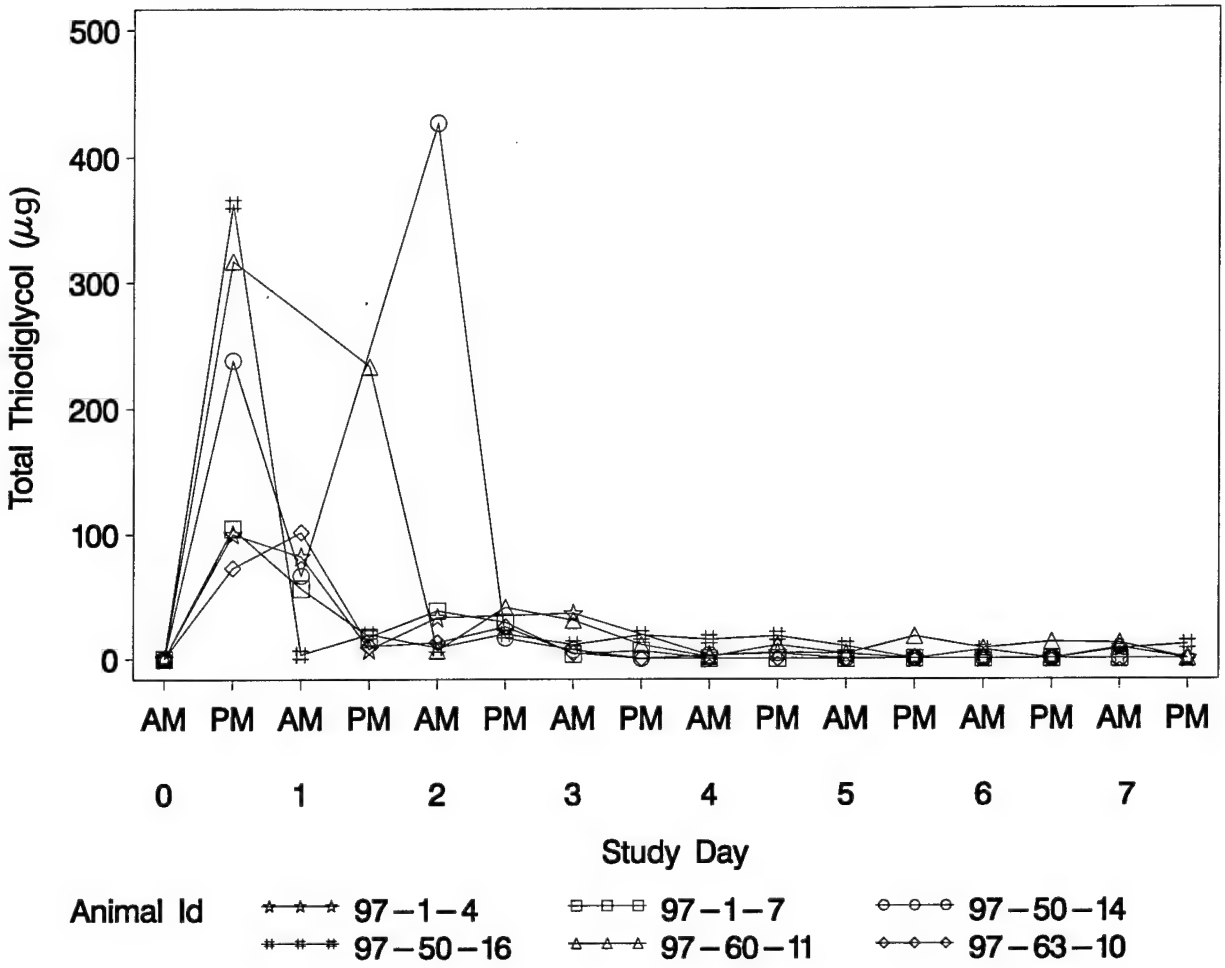


Figure 1. Total Thiodiglycol (μg) by Study Day of Six Different Animals Tested in Phase 1, Part B

concentrations, in general, peaked on day 0 for 4 of 6 animals when values were corrected for urine volume. One animal peaked on day 1 (AM) and the last animal peaked on day 2 (AM). The mean concentration of thiodiglycol on study day 0 (PM) and day 1 (AM) were significantly greater than the pretreatment mean on study day 0 (AM). Mean thiodiglycol concentration values were 0 on day 0 (AM) and peaked between 6 to 24 hr post exposure. Mean thiodiglycol concentration values peaked at a maximum mean value of 2.14 μg/mL at the earliest time point tested (6 to 8 hours post exposure), after which they declined to near 0 by the end of the study. The mean thiodiglycol concentration value decreased to 1.57 μg/mL by the morning of day 1, and by evening it had reduced to 0.27 μg/mL. Thiodiglycol levels remained detectable and low for the remainder of the 7-day observation. No significant differences were observed among other study day times.

5.0 CONCLUSIONS

5.1 Model Development

In Phase I, a full-skin-thickness SM-induced wound with an average size of 1406 mm^2 (Table 4 in Appendix D Statistical Report dated September 30, 1997 of Letter Report in Attachment B) was developed in weanling swine. The procedures and method to produce these lesions are described in Summary Report 1 of Attachment C. Day 2 was chosen to debride damaged sites based on articles describing casualty wounds and treatments from the Iran-Iraq war and USAMRICD directions for development of therapies.[3,4]

Histopathologic endpoints that were consistently reported on day 2 evaluations were epidermal necrosis, follicular necrosis, dermal neutrophil infiltration, subcutaneous hemorrhage, subcutaneous edema, and subcutaneous neutrophil infiltration. Clinical observations within and between animals were more variable than histopathologic endpoints. Erythema scores tended to be the most severe. Efforts to combine individual clinical observation scores to create an overall wound severity score resulted in erythema accounting for approximately half of the score. Clinical observations should be evaluated independently and not combined. As new technologies develop, quantitative methods for measuring clinical endpoints should be evaluated to reduce variability in the data. Variability was noted between individuals making clinical

observations. Maintaining the same evaluator throughout a study would be preferable. The anterior wounds appeared less severe than the posterior wounds. Overall site effect was statistically significant for WS, exudate, and edema. WS and edema scores were significantly greater on posterior sites than anterior sites. However, exudate scores were the opposite, probably the result of the animal's ability to kick at the anterior sites easier than posterior sites when irritation occurs. Treatments need to be rotated through sites to reduce bias. Wound size measurements were consistent, particularly on day 2 evaluations, and variability increased with time. Much of the variability was the result of different evaluators.

Using the same criteria to evaluate clinical observations for both wound development and wound healing, wound healing scores decreased over time. This does not mean that healing was occurring. This indicated that as the wound healed, the same scoring technique was confusing and not always appropriate, and as a result, two separate evaluations were created. The first evaluation was for wound development and emphasized wound severity and wound depth. The second evaluation was for wound healing and emphasized the amount of granulation tissue, neovascularization, epithelialization, presence of infection, wound size, and wound contraction. These evaluations were used with the last 6 animals of Phase I and Phase II animals.

5.2 Part A, Bandaging and Debridement Techniques

The methods of debridement and bandaging used in Phase II were as follows. A partial-skin thickness autograft was taken from the rump with the dermatome setting at approximately 0.75 mm depth. The dermatome was set at an approximately 1-mm depth to debride graft sites for Phase I, Part B, and Phase II. Grafts were attached using surgical staples (Butler, Columbus, OH). Bandaging of sites was performed by application of Thermazine® (Johnson and Johnson) to the graft sites only after graft application. Two 4x4 folded dry gauze sponges were placed over the non-treated sites and Xeroform™ gauze was placed over each grafted site. Mineral oil-soaked cotton was placed over the graft sites and sufficient dry 4x4 gauze folded over these sites to form a uniform layer across the three sites per side. A thin plaster-of-paris mold was placed over the gauze covering the three sites. A tissue adhesive, Skin Bond® (Smith and Nephew, Inc., Largo, FL), was applied around the mold and two adhesive Telfa™ 4x8 bandages were used to

secure the plaster-of-paris mold. Once both sides had been bandaged, 4-in elastic adhesive bandage secured the Telfa™ bandage. Vetrap™ was placed around the animal's torso, covered by stockinette, and secured with adhesive surgical tape. Table C-1 in Attachment C is a summary of the bandaging technique.

5.3 Part B, Characterization of Full-Skin-Thickness SM-Induced Burns

5.3.1 Clinical Observations and Histology

The SM-induced burn produced by day 2 in the weanling pig model using 400 µL undiluted SM applied percutaneously to the ventral abdomen for 2 hr was a severe wound. Histopathology confirmed a full-skin-thickness wound had been produced, as discussed in Letter Report 1 in Attachment B. Clinical observations indicated that minimal exudate was observed after SM exposure. The anterior wounds had an increased incidence of exudate than was seen in other lesions. This may be related to the animal's ability to reach these lesions with rear limbs. SM is reported to produce a burning and itching sensation.[1-14, 16-19] Some animals were seen trying to kick at their wounds, thus damaging the anterior wound sites. Not all animals appeared to exhibit the same degree of irritation, and not all anterior sites were observed with exudate.

Erythema and edema were prevalent at day 2 and were rated at near maximum severity. Necrosis of the lesions was observed to be of moderate or less severity. As the wounds progressed through day 8, the lesions were observed to increase in severity of necrosis. Wounds were observed to increase in severity from anterior to posterior wound sites. Swine skin becomes thinner as you move ventrally from the dorsum, and skin thickness in areas of the axilla and groin is thinner.[27,28] These areas provide less of a barrier to SM than the dorsal surface.

Histopathology confirmed the development of consistent full-skin-thickness SM-induced lesions in weanling pigs, and evaluated wound severity, depth of lesions, and evidence of wound healing. Histopathologic assessment of epidermal ulceration and necrosis in the epidermis, dermis, basal epithelium, and subcutaneous day 2 tissues was valuable for grading wound severity and depth during development.

5.3.2 Clinical Chemistry

Serum alkaline phosphatase declined over the course of the study, however this decline was within the parameter's range of normal and apparently not clinically significant. Alanine transaminase became elevated, and was possibly related to erythrocyte hemolysis in the area of the burn. Elevation of alanine aminotransaminase is generally associated with necrosis of hepatocytes, erythrocytes, myocardium or skeletal muscle. Evaluation of lactate dehydrogenase and creatine kinase (CK) indicated no significant damage to skeletal or cardiac muscle. One animal showed an elevated CK on day 7, primarily due to an elevated MM isoform increase, indicative of muscle damage. This muscle damage may be the result of multiple procedures (injections and biopsies) rather than SM-induced damage. The BB isoform of CK, present in brain, smooth muscle, thyroid, and prostate, showed increases by day 1 to 3 in 4 of 6 animals. These animals were anesthetized nearly every day for evaluation and sample collection, and therefore it is more likely the elevation is a result of anesthesia rather than SM induced. A slight increase was observed in BUN values by day one, however the increase is within the normal range. The BUN:creatinine ratio showed a slight increase, but again did not appear to be clinically significant. No significant changes were noted in any of the other clinical chemistry parameters measured.

5.3.3 Hematology

Increases in white blood cell counts were attributed to neutrophilia. An occasional rise in lymphocyte numbers was observed. Increases in neutrophils and lymphocytes are likely the results of stress, SM-induced damage, the wound healing process, and secondary infections of cutaneous lesions. Leukopenia, as reported previously by Gold and Scharf, [37] was not observed in hairless guinea pigs. Trends in decreasing hematocrit, hemoglobin, red blood cell counts, and red blood cell indices (mean corpuscular concentration) were slight and attributed to hemolysis at the SM-lesion and the multiple blood samples collected. Platelet counts decreased, particularly on day 1 and 3. Multiple venipunctures necessary for blood collection could result in reduced platelets. No significant changes were observed in other hematology parameters measured.

5.3.4 Urinalysis and Thiodiglycol Analysis

Blood was present in the urine and peaked by day 3, then declined during the remainder of the study. This is consistent with the hemolysis noted at the dosed sites. Elevated protein (albumin) levels in the urine occurred by day 3 and remained elevated throughout the 7-day observation period. Urine volume increased slightly toward the end of the study. Recovery from anesthesia during wound development on days 0 and 1, and an increased frequency of anesthesia for data collection early in the study, would decrease water intake and thus reduce the amount of urine being eliminated early in the experiment. As the animal was able to drink more, her urine volume increased. Nitrites/nitrates in the urine were not detected in 83 percent of the animals on day 0, but were present in all animals by day 7. Nitrites/nitrates are associated with fecal contamination and microbial activity of gram negative bacteria. Leukocytes were not found in any urine sample and urine cultures were not performed. Increased nitrites/nitrates detected probably were the result of fecal contamination of the urine. No significant changes were noted in any of the other urinalysis chemistry parameters measured.

Thiodiglycol is a major metabolite of SM, and is eliminated in the urine. SM is biotransformed by direct alkylation reactions with tissue components, chemical hydrolysis, or conjugation products such as with glutathione. Urine volume was collected twice a day and a 10 mL sample from each collection sent to USAMRICD for thiodiglycol analysis using gas chromatography/mass spectrometry with negative ion chemical ionization. Total thiodiglycol was tested, which included free plus acid-labile conjugates. Thiodiglycol was present in urine samples at the earliest time point tested (6 to 8 hr) with levels ranging from 0.66 to 4.98 ng/mL and a mean of 2.14 ng/mL. Peak levels were observed in half of the animals at this earliest time point of 6 to 8 hr and between 24 and 48 hr for the remaining animals. The mean level of thiodiglycol by the evening of day 3 had declined to 50 ng/mL. The mean levels were between 10 and 40 ng/mL for the remainder of the 7 day observation period.

Full-skin-thickness SM-induced burns were obtained at all six sites on the ventral abdomen of female Yorkshire and Yorkshire cross weanling pigs. Histologically, the wounds were fully developed and most severe by day 8 or 9. These wounds induced a neutrophilia over the seven-day observation period, localized hemolysis, and urinary excretion of the breakdown products of the cutaneous lesion (protein, red blood cells, and hemoglobin).

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Task 94-33 Phase II

Efficacy Evaluation of Dermagraft-TC™ and Autografts on Sulfur-Mustard-Induced Full-Skin-Thickness Lesions in the Weanling Swine

Phase II evaluated the temporary wound dressing, Dermagraft-TC™ (DGTC), and autograft treatments of full-skin-thickness SM-induced burns in weanling swine.

1.0 INTRODUCTION

Found in Task 94-33 Phase I Report.

2.0 OBJECTIVES

Found in Task 94-33 Phase I Report.

3.0 MATERIALS AND METHODS

Found in Task 94-33 Phase I Report.

3.1 Model Development

Found in Task 94-33 Phase I Report.

3.2 Experimental Design

A total of thirty-one Yorkshire, female swine from Isler Genetics, Inc (Prospect, Ohio) were used in Phase II. Results from twenty-four animals were used in assessing DGTC and autograft effects on healing of full-skin-thickness, SM-induced burns. The data from seven remaining animals were not included in the assessment because of health problems. Tables I-1 (Attachment I) and I-2 summarize the health problems of Phase II animals. The test system used

in this phase is described under the Phase I section. The weight range for the animals used in this section was 6.4 to 12 kg. The burn lesions were produced as described in Attachment C Summary Report C-1. Additional or modified procedures to those described in Attachment C are described below. Treatments were applied contralaterally and rotated anterior to posterior to prevent site location bias.

One animal per day was dosed with two dosing days per week. The second animal was not prepared or dosed until the first animal was proven decontaminated, using the MINICAM™, and removed from housing in the chemical fume hood. The chemical fume hood and caging were cleaned and prepared for dosing the second animal. Two days after dosing, the lesions on each animal were clinically evaluated, and a dermatome was used to remove an approximately 1 mm thick layer of damaged skin from four of the six lesions. An example of the clinical evaluation forms (wound development and wound healing evaluations) and definitions of scoring designations are in Attachment H. The treatment allocation is provided in Table 1.

Table 1. Phase II Design for Treatment Rotations

	ROTATION 1	ROTATION 2	ROTATION 3
Anterior Design Posterior	A and B C and D E and F	E and F A and B C and D	C and D E and F A and B
Animal Nos	1, 1a, 4, 4a, 7, 10, 13, 16, 19, 19a, and 22	2, 2a, 5, 8, 11, 14, 17, 17a, 20, and 23	3, 3a, 6, 9, 12, 15, 18, 21, and 24

SM dose confirmation samples were within 10 percent of the expected concentration values, except for four samples that were analyzed in February and March of 1997. The concentrations of these samples were between 87 and 89 percent of the expected values. Full-skin-thickness burns were created on these dosing days. Evidence supporting the full-skin-thickness burns are 1) biopsies taken on day 2 from similarly treated animals showed a full-skin-thickness burn, and 2) animals surviving 38 days showed no hair growth within the lesion area, indicating follicular cell death in the deep dermis and subcutaneous tissues, and implying a full-skin-thickness burn.

3.3 Treatments

Day 2 was chosen to debride 4 of the 6 burn sites and apply graft treatments. This 2-day time, based on articles describing casualty wounds and treatments during the Iran-Iraq war and USAMRICD directives for development of therapies[1,2] was estimated to be the maximum time needed to transport soldiers to a treatment facility, Three wound treatments were tested: no treatment and no debridement, autograft (debrided) and DGTC (Advanced Tissue Sciences, La Jolla, CA) temporary wound dressing (debrided). Prior to debriding treatment sites, a partial-skin-thickness autograft approximately 0.75 mm thick was removed from the dorsal-lateral aspect of the rump of each animal and placed on ice in a sterile pyrex glass dish containing refrigerator-cooled RPMI media until used (< 1 hr). Four sites were debrided using a compressed nitrogen gas (Praxair, Columbus, Ohio) driven dermatome (Zimmer Air Dermatome, Zimmer, Inc. Columbia, MD). After debridement, the swine autograft and DGTC were each applied to two debrided sites. DGTC is a human fibroblast-derived temporary skin substitute consisting of a polymer membrane (silicone) coated with porcine dermal collagen and neonatal human fibroblast cells cultured under aseptic conditions *in vitro*.[33] DGTC was stored at ≤ 70 C and used within 1 hr (0.15-1 hr) of thawing in a 37 C water bath. Grafts were secured using staples (35 wide, Staple Pistol Grip, Butler, Columbus, OH).

Exposure sites were bandaged after application of the grafts. Bandages were changed on Mondays, Wednesdays and Fridays, but not earlier than two days after grafting. This schedule was maintained for approximately 17 days. Bandaging techniques are described in the Phase I conclusion section under Part A, Debridement and Bandaging. No biopsies were taken on day 2 of the efficacy study to avoid disturbing the grafts. Clinical evaluations of lesions were made on approximately day 2, 10, 17, 24, 31 and 38. Clinical parameters evaluated in this experiment were adherence, contraction/closure, durability, edema, epithelialization, erythema, eschar, exudate, granulation, inflammation, necrosis, rejection, wound size, and vascularization. Clinical observations were conducted using the definitions and scoring presented in Attachment H, entitled Clinical Observation Evaluation/Definition 2 dated 3-28-97. Contraction measurements were based on a subjective assessment of area of the wound involved and not an objective

measurement of degree of contraction as measured in Phase III (definition of assessment in Attachment H). Discussions with the TPOC and COR resulted in a quantifiable method for contraction assessment in Phase III (see difinition in Attachment H, Clinical Observation/Evaluation/Definition 3 Worksheet). Animals were anesthetized on study day 38 for clinical evaluation of wounds. The lesions were excised, and animals then euthanatized. Histopathologic evaluations were conducted on day 38 lesions for depth of necrosis, presence or absence of necrosis of basal epithelium or ulceration (loss of epidermis), granulation tissue response, and re-epithelialization (hyperplasia).

3.4 Microorganisms in SM Burn Lesion

USAMRICD requested sampling of each lesion and a neutral site (shoulder and/or ventral abdomen) on the last two animals of Phase II. Samples were collected from each lesion and from a neutral site of animal 97-131-11 and animal 97-54-11 three days before the observation period ended. A sterile swab (Baxter Healthcare Corporation, Deerfield, IL) was rubbed over each lesion and then placed in commercially-prepared sterile media. These cultures were evaluated by The Ohio State University Diagnostics Laboratory. A basic identification of any organism with an indication of quantity (few, moderate, many) was requested. Organisms identified were from surface contamination, since swabs were taken from the surface of the wound and not from a punch biopsy of deeper tissues.

3.5 Statistical Methods

The Phase II Statistics Report is in Attachment E. The efficacy of DGTC and pig skin autograft treatments compared to no treatment and to each other was evaluated by analyzing the histopathologic endpoints and clinical observations data.

3.5.1 Wound Development

Some statistical comparisons were based on the assumption that Phase II wounds were

similar to Phase I, Part B wounds. Clinical observation data from Phase I, Part B and from Phase II were used to determine if wounds from these two phases were similar on day 2. Comparisons between the two data sets were made using two sample t-tests (SAS PROC TTEST) and plots.

3.5.2 Histopathology

Analysis of variance (ANOVA) models were fitted to depth of necrosis, granulation tissue response, and re-epithelialization for day 38 data. These parameters were selected to determine the efficacy of wound treatment and to estimate animal-to-animal variation. Appropriate contrasts were used to assess whether there were differences between wound treatments. The SAS (V6.12) MIXED procedure was used to fit the ANOVA models. A logistic regression model was fitted to the ulceration data (scored as present/absent) using the SAS (V6.12) GENMOD procedure.

Since histopathology was evaluated only on day 38 lesions in Phase II, day 2 data from Phase I, Part B were combined with Phase II data to estimate wound severity and degree of wound healing over time. The SAS (V6.12) MIXED procedure was used to fit ANOVA models to the combined data for each endpoint, and appropriate contrasts were used to compare day 2 and day 38 histopathology endpoints.

Summary tables were prepared for histopathologic indicators and displayed number of observations, mean, standard deviation, minimum, maximum, and percent incidence. Mean scores for histopathological endpoints and observed values were plotted for each treatment group.

3.5.3 Clinical Observations

ANOVA models were fitted to clinical observation scores and included the effect of wound treatment, the effect of study day, an effect due to the interaction between wound treatment and study day, and a random animal effect. Pairwise comparisons between wound treatments were carried out using appropriate contrasts in the analysis of variance model (SAS

PROC MIXED).

Summary tables prepared for clinical observation data displayed number of observations, mean, standard deviation, minimum and maximum. Mean scores and their associated 95 percent confidence intervals for each wound treatment group were also graphically displayed.

3.5.4 Microorganisms on SM Burn Lesions

Frequency of the most common microorganism colonies were reported along with a summary of microorganisms observed.

4.0 RESULTS

Twenty-four weanling pigs with six sites per animal were used to compare the efficacy of DGTC, pig skin autograft, and no treatment in treating SM-induced dermal lesions. Two sites per animal were assigned to each treatment.

4.1 Wound Development

Descriptive statistics and two sample t-test results comparing Phase II and Phase I, Part B clinical signs on day 2, are presented in Table 1 of the Phase II Statistics Report in Attachment E. Statistically significant differences between Phase I, Part B and Phase II clinical observations existed for erythema, edema, necrosis and wound size. Figures 1-5 in Attachment E illustrate the differences in the clinical observation scores between Phase II and Phase I, Part B. Since there appeared to be significant differences in wound development between Phase I, Part B and Phase II, the results of analyses combining data from both phases should be interpreted with caution. Statistically significant differences reported between Phase I Part B and Phase II clinical observation scores on day 2 for erythema, edema, necrosis, and wound size may be due to the following reasons: 1) Animal Conformation – SM application on deep-chested animals is not as uniform as that on round-chested animals. The thoracic surface has a steeper angle on deep-

chested animals, 2) Dosing site location – Anterior sites on deep-chested animals are generally smaller and less uniform than posterior sites on the same animal and between animals. This is due to the flat abdominal surface versus the angled thoracic surface for SM application, 3) A larger n in Phase II – A larger number of animals were used in Phase II (31) than Phase I Part B (6). A greater variation in animal size and shape was observed in the Phase II animals than those of Phase I Part B, 4) Different evaluators – The primary evaluator was not available for all observation times over the course of the study (Jan 1997 through Aug 1997). Scoring may be slightly different between evaluators for the same observation, since observation scores are subjective, 5) Different SM lots – Over the course of the study, several lots of SM were used. Purity varied, but was within the range of acceptability (within 20 percent) established by Battelle Chemists. In summary, clinical observation evaluations are not consistent over time and should not be used as a primary endpoint in future studies. However, clinical observations scores are valuable assessments in describing the visual interpretations of the wound.

4.2 Histopathology

Table 2 in Attachment E presents summary statistics for histopathological indicators on study day 38. As indicated in Table 2, necrosis of the basal epithelium was not present in any of the sites examined for any treatment. Ulceration (scored as present/absent) was observed in 39 percent of no treatment sites, compared to 4 percent of autograft and 18 percent of DGTC sites. Re-epithelialization was nearly complete in autograft sites, where the mean score (3.96) was near maximum. Figures 6-9 of the Phase II Statistics Report in Attachment E present the mean depth of necrosis, ulceration, granulation tissue response, and re-epithelialization scores, respectively, overlaid on observed values for the three treatment groups.

Results of ANOVA models fitted to the depth of necrosis, granulation tissue response, and re-epithelialization scores on day 38 are presented in Table 3 of the Phase II Statistics Report in Attachment E. No differences between treatment groups were noted in the analysis of depth of

necrosis and granulation tissue response. Animal-to-animal variability was highly significant for these endpoints. Re-epithelialization scores were significantly greater in autograft sites compared to no treatment and DGTC sites. The difference between no treatment and DGTC was not statistically significant. Table 3 of Attachment E also provides the results of the logistic regression model fitted to the ulceration data. Ulceration was present in a significantly greater proportion of no treatment sites compared to autograft and DGTC sites. Also, ulceration was present in a significantly greater proportion of the DGTC sites compared to autograft sites. No statistical models were fitted to necrosis of basal epithelium as it was not present in any of the sites examined.

Results of the statistical comparisons of day 2 and day 38 histopathological endpoints for each wound treatment are displayed in Table 4 of Attachment E. Granulation scores were significantly higher on day 38 than on day 2 for untreated, autograft and DGTC sites. Dermal depth of necrosis did not show any marked difference between day 2 and day 38 for any wound treatment. Ulceration was absent and epithelium was not lost for all animals on day 2 in Phase I, Part B, and therefore comparisons were not carried out for ulceration and re-epithelialization scores.

4.3 Clinical Observations

Descriptive statistics for all clinical observation endpoints (definitions and scoring described in Attachment H, Clinical Observations Evaluation/Definition 2 dated 3-28-97) are presented in Table 5 of the Phase II Statistics Report in Attachment E. From this table, it is evident that erythema, edema, and necrosis peaked early in the study for all treatment groups, whereas other clinical signs that are more indicative of healing, such as epithelialization, neovascularization, and granulation, peaked or were evident later in the study. Eschar and exudate were observed throughout the observation period until the lesion healed. Infection was rare and observed (scabs had been removed) only in a small number of sites on days 10 and 17. Contraction scores appeared to be greater for no treatment sites. Adherence, durability, and rejection could not be evaluated for untreated sites or for the wounds that were completely healed. Figures 10-23 of the Phase II Statistics Report in Attachment E present mean clinical

observation scores with 95 percent confidence intervals plotted against time for each treatment. It is evident from these figures that for most of the clinical observation parameters, the wounds healed more rapidly at autograft sites, compared to the other treatments, and that DGTC sites healed more rapidly than no treatment sites.

The results of the random effect model fitted to clinical observation parameters to assess animal-to-animal variability and the effect of wound treatment over time are summarized in Table 6 in Attachment E. The overall study days effect and animal-to-animal effect were statistically significant for all clinical observation parameters. A significant difference due to wound treatment was observed for all parameters except erythema, exudate, granulation and infection. Wound severity scores (eschar, necrosis, and wound size) tended to be significantly lower for autograft sites than untreated and dermagraft sites. Wound size is not a good indicator of healing as a single endpoint. A decreasing WS evaluates only the degree of reepithelialization that is observed. Assessment of the wound below the epidermis is not considered. The degree of granulation, contraction, and neovascularization of deeper dermal layers may not occur at the same rate as the surface reepithelialization and is not reflected in the WS score. Wound healing scores (contraction, epithelialization, and vascularization) showed that wounds healed significantly faster at autograft sites than at untreated and dermagraft sites and that dermagraft sites healed faster than untreated sites. Graft evaluation scores (adherence, durability, and rejection) were significantly lower for autograft sites than dermagraft sites. Significant interactions between study day and wound treatment effects indicate that the main trend varied over time. These effects are best seen in Figures 10-23 of the Phase II Statistics Report in Attachment E. For example, adherence scores on study day 10 appeared to be greater for autograft than dermagraft sites, while the opposite was observed on later study days (Figure 10). The interaction effect was not significant for contraction and, as seen in Figure 11 of the Statistics Report, the relationship between treatment scores is consistent over time.

4.4 Microorganisms in SM Burn Lesions

Table 2 summarizes the presence of the most common microorganisms: anaerobic gram positive rods (Anrod g+), Echericia coli (E coli), Enterococcus (Enter), Moraxella (Morx), Staphylococcus aureus (Staph aureus), Staphylococcus hyicus (Staph hyicus), Streptococcus equisimilis (Str eq). Each of these microorganisms was found in multiple sites on both animals, usually on more than one day. Anrod g+ and Staph aureus were present on all sites on at least one day and on every wound site on the last day. Staph aureus was also present in neutral sites on the last day.

Several other microorganisms were noted: Actinomyces pyogenes (Act py), Bacillus (Baci), Echericia coli lactose negative (E coli LN), Echericia coli mucoid (E coli mu), Non Fermenter (Non Fer), Pseudomonas (Pseudo), Staphylococcus coagulase negative (Staph CN), Staphylococcus intermedius (Staph inter), Streptococcus alpha (Str alpha), Streptococcus porcine (Str porc), and Streptococcus uberis (Str ub). These microorganisms were noted on a small number of sites and on one animal, and usually on one day.

Table 3 provides information on the presence and quantity of microorganisms found on each animal, site, and day. As shown in this table, numbers of E coli and Staph aureus tended to be greatest over a longer period, compared to the other organisms. In addition, multiple strains of bacteria were noted in some sites. When present, very few, few, or light colonies were observed on the neutral sites, even when many colonies were observed on the wound sites. Quantity of colonies were not reported for Anrod g+ bacteria.

Table 2. The Presence of Most Commonly Found Microorganisms in Two Animals During Phase II Wound Healing Experiments

Animal	Study Day	Site	Presence of Microorganism						
			Anrod g+	E coli	Enter	Morx	Staph aureus	Staph hyicus	Str eq
97-130-11	8/1	Wound	3/6	3/6	1/6	0/6	5/6	2/6	2/6
		Neutral	Yes	No	Yes	No	Yes	No	No
	8/4	Wound	5/6	3/6	2/6	2/6	6/6	1/6	4/6
		Neutral	Yes	No	Yes	No	No	No	No
	8/6	Wound	6/6	2/6	5/6	1/6	6/6	3/6	3/6
		Neutral	Yes	No	Yes	No	Yes	No	No
97-54-11	8/1	Wound	1/6	0/6	0/6	0/6	1/6	1/6	0/6
		Neutral	Yes	No	Yes	No	No	Yes	Yes
	8/5	Wound	5/6	2/6	4/6	3/6	4/6	3/6	4/6
		Neutral	No	No	No	No	No	No	No
	8/6	Wound	6/6	0/6	5/6	0/6	6/6	6/6	4/6
		Neutral	Yes	No	No	No	Yes	Yes	Yes

Table 3. Presence of Microorganisms Identified By Site and Observation Day in Two Animals During Phase II Wound Healing Experiments

Actinomyces pyogenes = Act py
Anaerobic gram positive rods = Anrod g+
Bacillus = Baci
Echericia coli = E coli
Echericia coli lactose negative = E coli LN
Echericia coli mucoid = E coli mu
Enterococcus = Enter
Moraxella = Morx
Non Fermenter = Non Fr
Pseudomonas = Pseudo
Staphylococcus aureus = Staph aureus
Staphylococcus coagulase negative = Staph CN
Staphylococcus hyicus = Staph hyicus
Staphylococcus intermedius = Staph inter
Streptococcus alpha = Str alpha
Streptococcus equisimilis = Str eq
Streptococcus porcine = Str porc
Streptococcus uberis = Str ub

Key of Comments (colonies found on Agar plates)
Few colonies = F
Very few colonies = VF
Many colonies = Many
Moderate colonies = Mod
Light colonies = ligh

Animal Number	Submission Date	Microbiology Number	Lesion Site	Act py	Anrod g+	Baci	E coli	E Coli LN	E coli mu	Enter	Morx
97-130-11	08/01/1997	972855	A		Yes		Mod	Mod			
			B								
		972849	C		Yes		Many		Many	VF	
		972853	D								
		972848	E				Mod				
		972850	F		Yes						
		972852	Shoulder		Yes					VF	
	08/04/1997	972884	A		Yes		Many – 2			Many	Many
		972885	B		Yes						
		972886	C		Yes		Many – 2				
		972887	D								
		972888	E		Yes		Mod				
		972889	F		Yes						
		972883	Shoulder		Yes					F	

Table 3. Presence of Microorganisms Identified By Site and Observation Day in Two Animals During Phase II Wound Healing Experiments (Continued)

Animal Number	Submission Date	Microbiology Number	Lesion Site	Act py	Anrod g+	Baci	E coli	E Coli LN	E coli mu	Enter	Morx
97-130-11	08/06/1997	972938	A		Yes					VF	Mod
		972939	B		Yes					VF	
		972940	C		Yes		Mod			VF	
		972941	D		Yes					VF	
		972942	E		Yes		Many – 2				
		972943	F		Yes					VF	
		972944	Neutral Site							VF	
97-54-11	08/08/1997		A								
			B								
			C								
			D								
			E								
		972851	F	Mod	Yes						
		972854	Shoulder		Yes					VF	
	08/05/1997	972908	A		Yes						
		972909	B		Yes					Many	
		972910	C		Yes					Many	Many
		972911	D		Yes		Mod				
		972912	E							Mod	Mod
		972913	F		Yes		Mod			Mod	Mod
		972915	Shoulder			Mod					
	08/06/97	972945	A		Yes					Mod	
		972946	B		Yes					VF	
		972947	C		Yes					VF	
		972948	D		Yes					VF	
		972949	E		Yes					VF	
		972950	F		Yes						
		972951	Neutral Site		Yes						

Table 3. Presence of Microorganisms Identified By Site and Observation Day in Two Animals During Phase II Wound Healing Experiments (Continued)

Animal Number	Submission Date	Microbiology Number	Lesion Site	Non Fer	Pseudo	Staph aureus	Staph CN	Staph hyicus	Staph inter	Str alpha	Str eq	Str porc	Str ub
97-130-11		942855	A			Mod					VF		
			B										
		972849	C										
		972853	D			Many		Many					
		972848	E			Mod - 2		Mod					
		972850	F			Mod					VF		
		972852	Shoulder			Many - 3							
	08/04/1997	972884	A			VF			VF				
		972885	B			Many							
		972886	C			Many					Many		Many
		972887	D			Many					Many		
		972888	E			Mod		Mod			Mod		
		972889	F			Mod					Mod		
		972883	Shoulder			Many							
	08/06/1997	972938							F				
		972939	A			Mod							
		972940	B			Mod - 2							
		972941	C			Mod		Mod			Mod		
		972942	D			Many - 2		Many			Many		
		972943	E			Many - 2					Many		
		972944	F			Many		VF					
97-54-11	08/01/1997		Neutral Site			VF							
			A										
			B										
			C										
			D										
		972851	E										
		972854	F			Mod		Mod					

Table 3. Presence of Microorganisms Identified By Site and Observation Day in Two Animals During Phase II Wound Healing Experiments (Continued)

Animal Number	Submission Date	Microbiology Number	Lesion Site	Non Fer	Pseudo	Staph aureus	Staph CN	Staph hyicus	Staph inter	Str alpha	Str eq	Str porc	Str ub
	08/05/1997	972908	Shoulder					F		F	F		
		972909	A			Many - 2					Many		
		972910	B			Many							
		972911	C			Many		Many			Many		
		972912	D			Mod		Mod			Mod		
		972913	E									Mod	
		972915	F		Mod			Mod			Mod		
	08/06/1997	972945	Shoulder	Mod	Mod - 2		Mod						
		972946	A			Mod		Mod			VF		
		972947	B			Mod		Mod			VF		
		972948	C			Many - 2		Many					
		972949	D			Many		Many					
		972950	E			Mod	VF	Many			VF		
		972951	F			F		Mod			VF		
			Neutral Site			F	VF	Light			F		

5.0 CONCLUSIONS

Overall, pig skin autograft sites healed faster and wounds at 38 days were less severe compared to DGTC and no treatment sites. In addition, DGTC sites healed more rapidly than no treatment sites. Treatments were rotated through anterior to posterior sites on the ventral abdomen. This design reduced the bias of increasing wound severity from anterior to posterior sites on the ventral abdomen noted during model development.

Autograft and temporary wound dressing adherence at graft sites was incomplete for most lesions across all animals evaluated. Necrosis at the center of the lesion was noted in most grafted lesions by day 10 (46 of 48 for autograft sites and 25 of 48 for DGTC). During healing, various portions of the grafts adhered to the dermatomed area by day 7, particularly at the periphery of each lesion for the autografts. Bandages were removed by day 17 and non-adhered autografts had generally sloughed by this time. DGTC remained adhered for longer periods and was either found in the bandaging material or in the caging when the scabs were sloughed. Since portions of the autografts did not adhere to the prepared wound bed, the damaged tissue was not completely removed by the dermatome method of debridement. This finding was supported by the histopathology on day 38 for both graft groups (epithelial growth over granulation tissue filled in the dermal defect). The dermatome, set at its thickest setting (1 mm), did remove necrotic tissue from the periphery of the wound, but not from the center. The dermatome used in a single pass for debriding full-skin-thickness wounds is not advocated. If it is to be used, numerous passes will need to be made; surgical debridement or an alternative method (enzyme or laser debridement) probably would be more economical and efficient.

Statistically significant differences reported between Phase I Part B and Phase II clinical observation scores on day 2 for erythema, edema, necrosis, and wound size may be due to the following reasons: 1) Animal conformation – SM application on deep-chested animals is not as uniform as that on round-chested animals. The thoracic surface has a steeper angle on deep-chested animals than round-chested ones, 2) Dosing site location – Anterior sites on deep-chested animals were generally smaller and less uniform than posterior sites on the same animal and between animals. This is due to the flat abdominal surface versus the angled thoracic surface for

SM application, 3) A larger n in Phase II – A larger number of animals were used in Phase II (31) than in Phase I Part B (6). A greater variation in animal size and shape was observed in Phase II animals than those in Phase I Part B, 4) Different evaluators – The primary evaluator was not available for all observation times over the course of the study (Jan 1997 through Aug 1997). Scoring may be slightly different between evaluators for the same observation, since observation scores are subjective, 5) Different SM lots – Over the course of the study, several lots of SM were used. Purity varied, but was within the range of acceptability (within 20%) established by Battelle Chemists. In summary, clinical observation evaluations are not consistent over time and should not be used as a primary endpoint in future studies. However, clinical observation scores are valuable assessments in describing the visual interpretation of the wound.

The scoring system used for adherance, durability, and rejection of the graft during the course of the study appeared inadequate after the graft had either imbeded and was viable (autograft) or sloughed (autograft and DGTC). In Table 5 of the Phase II Statistics Report in Attachment E, it appears as if the grafts improve in these parameters over time for those lesions being evaluated. In addition, for the DGTC, it appears as if the TWD was not evaluated on day 31 (n=0), but begins sloughing in 6 sites on day 38. All 48 sites were evaluated, but there was not a designation for inability to score the lesion using the categories provided. For example, a few of the remaining TWD fibers would not be observable in the hard scabs that had formed, but by the next week could be seen along the edges of the lesion after the scab had sloughed. Future studies will incorporate a revised clinical observation procedure to address these issues.

Wounds were observed to split along the posterior edge of the wound. These areas appeared healed on gross observation, but a hematoma was in areas of incomplete healing in deeper tissue. Histopathology taken on day 38 indicated that necrosis in the dermal tissue was near maximum at all sites. Granulation/fibrotic tissue was filling in the defect, however it was incomplete by day 38. Since the dermatome did not remove all of the necrotic tissue, areas of incomplete healing are anticipated despite gross observation of epithelialization of the lesion.

Studies are planned to evaluate alternative methods of debridement. Once a debridement method is determined, the TWD will be reassessed. Pulsed carbon dioxide laser debridement has

been reported to provide improved healing in SM burns in weanling swine.[4] Sharp surgical tangential excision has been the method of choice to debride wounds.

The microorganisms identified on the lesions are those typically found in the air (Staphylococcus aureus) or from the gastrointestinal tract (Enterococcus, Echericia coli).[5] Most organisms found can be pathogenic. Since these microorganisms were identified in the last 3 weeks in one or more lesions per animal, these microorganisms may typically be observed in burns late in the healing process (as wound is drying). The microorganisms observed most frequently are those also reported in human beings.[5] Future studies should include identification and quantification of microorganisms at wound sites over the course of the study.

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Task 94-33 Phase III

Solubility and Stability Testing of Sulfur Mustard, and Characterization of Superficial Dermal and Full-Skin-Thickness SM-Induced Lesions Using Biomechanical Monitoring Techniques in the Weanling Pig Model

1.0 INTRODUCTION

Task 94-33 developed sulfur mustard (SM)-induced, full-skin-thickness burns at each of six ventral abdominal sites to test a temporary wound dressing (Dermagraft-TC™). A volume of 400 µL of undiluted SM was applied on day 0 to each of the six sites and left for 2 hr to produce full-skin-thickness burns. During the Iran-Iraq conflict, Kadivar and Adams reported that SM casualties, with the majority of the lesions being partial-skin-thickness burns, received hospital treatment by day 2.[1] It was estimated that it would take between 48 and 96 hr to deliver an exposed soldier from the battlefield to a properly equipped treatment facility. Therefore, day 2 was the focus for therapeutic regimens and pathophysiology of cutaneous lesions in the weanling swine burn model. The histopathologic definition of a full-skin-thickness burn includes damage to dermal structures and also may involve the panniculus muscle.[2] The histopathologic definition for a superficial dermal burn, as defined by Arturson[2], includes damage to the epidermis and the upper third of the dermis. To develop partial-skin-thickness burns, the same SM volume was used and the exposure time varied.

Leukopenia has been reported in human cases of severe systemic intoxication to SM, and may be a result from both direct action of the agent on hematopoietic tissue and from a secondary, bacterial infection facilitated by damage to skin and/or respiratory epithelium.[3] Changes in clinical pathology parameters following SM exposure have been noted in both hairless guinea pigs[4] and weanling pigs. As SM is systemically absorbed following cutaneous exposure, hematology and clinical chemistry examinations were conducted to determine if the systemic toxicity occurred.

A burn encompassing 20 percent or more of the body surface area is reported as life threatening by researchers at the Institute of Surgical Research (ISR) in San Antonio. In the weanling swine model, the burns covered less (approximately 2 percent) than 20 percent of the body surface area. The current model would require modifications to assess efficacy of

therapeutic regimens for wounds covering greater than 20 percent of the body surface area. The fourth part of Phase III was proposed to create full-skin-thickness and superficial dermal lesions covering greater than 20 percent of the body surface area, but was not funded as a part of Phase III. Thus, Phase III was designed to:

1. Evaluate various solvents to be used as diluents for SM for percutaneous design,
2. Chose an anesthetic regimen with minimal effects, or consistent vascular effects, on dermal dose sites,
3. Characterize the full- and partial-skin-thickness SM-induced wound using dermal monitoring equipment (Laser Doppler, ultrasound, Evaporimeter, and Minolta® Chroma Meter) and special histopathology staining and techniques, and
4. Assess systemic effects using selected clinical chemistries and hematology parameters.

2.0 OBJECTIVES

2.1 Part A, Solubility, Stability, and Hydrolysis Testing

The purpose of this portion of the study was to identify an appropriate SM diluent for providing solutions for cutaneous application using solubility, stability, and hydrolysis testing.

2.2 Part B, Anesthesia Effects

The purpose of this part was to choose an anesthetic regimen with minimal effects, or at least consistent vascular effects, on dermal dose sites. These evaluations were to identify an anesthetic regimen to be used in subsequent SM-induced skin burn studies at the United States Army Medical Research Institute of Chemical Defense (USAMRICD) and at Battelle's Medical Research and Evaluation Facility (MREF).

2.3 Part C, Development of Full-Skin-Thickness and Superficial Dermal Sulfur Mustard Burns by Varying the Exposure Time

Part C was to develop and characterize full-skin-thickness and superficial dermal burns in weanling swine, to determine if SM exposure time can be reduced and induce full-skin-thickness burns, and to assess systemic effects that may result from the percutaneous application of SM. Using current SM dosing techniques and dose volume, this experiment was designed to determine exposure time parameters for producing full-skin-thickness and superficial dermal burns. Wounds were characterized using histopathologic and immunohistochemical techniques. In addition, biomechanical engineering instruments were used to characterize the lesions using non-invasive techniques.[5-32] Systemic effects were evaluated using selected clinical chemistries and hematology. The anesthetic regimen identified in Part B was used. Each wound was characterized using specified equipment, clinical evaluations, histopathology, and immunohistochemical endpoints described in the Material and Methods section for Phase III below. Additional control sites near the dosed sites on the ventral abdomen and on the side of the SM-exposed animals were evaluated to determine if percutaneous SM exposures caused systemic effects which affect the control sites of these animals.

3.0 MATERIAL AND METHODS

3.1 Part A, Solubility, Stability, and Hydrolysis Testing Experimental Design

The Chemistry Group at Battelle's MREF was tasked to evaluate the stability of SM in the following four liquids: peanut oil, propylene glycol, polyethylene glycol (PEG) 200 and PEG 400. This preliminary work was needed to assess the possibility of dosing SM mixtures over the 25 to 100 percent concentration range. Details on the equipment used the results of testing, and conclusions for stability, solubility, and hydrolysis testing can be found in Attachment G. The stability of SM in mixtures were summarized in two reports and are in Attachment G. To evaluate the stability of the SM liquids, an organic chromatographable solvent was needed. Methylene chloride, hexane and chloroform were tested. Methods and results for these solvents are in Attachment G Chemistry Reports.

3.2 Part B, Anesthesia Effects Experimental Design

General anesthesia causes a decrease in blood flow to the skin and may interfere with the evaluation of effects of a vesicant on the skin. Different anesthetic regimens were tested to evaluate their effect on the skin. SM was not applied. Table I-4 in Attachment I identifies animals used in Part B.

Six female weanling Yorkshire or Yorkshire cross pigs were used to evaluate anesthetic regimens using a modified cross-over design with a one-week washout between treatments. The cross-over design was modified when the sponsor requested substitution for an anesthetic regimen that was determined to be inefficient and unsafe. The following anesthetic regimens were used:

Regimen X - 0.044 mL/kg of a mixture of Telazol® (250 mg of tiletamine and 250 mg zolazepam, (Elkins-Sim, Inc., Cherry Hill, NJ) reconstituted with 5 mL of a 100 mg/ml xylazine chloride solution (Ben Venue Laboratories, Inc., Bedford, OH) in repeated intramuscular (im) injections (Telazol/Xylazine).

Regimen Y - Telazol®/xylazine solution as a pre-anesthetic and inhalation of isoflurane to maintain anesthesia.

Regimen Z - Isoflurane (Solvay Animal Health Inc., Mendeta Heights, MN) inhalation only (Isoflurane).

Regimen W - Separate im injections of ~20 mg/kg body weight of a 100 mg/mL ketamine solution (Ketaset®, Fort Dodge, IA) and ~2 mg/kg of a 100 mg/mL xylazine solution (Ketamine/Xylazine). Repeated,. separate injections were administered as indicated.

Anesthetic regimen Z was stopped after the first week because the time to anesthetize and evaluate the first two animals exceeded the desired 2 hr time limit for anesthesia and evaluations. Anesthetic regimen W was substituted for regimen Z to evaluate three anesthetic regimens. Table 1 displays the modified cross-over design used in Part B of Phase III with X, Y, Z, and W representing the anesthetic regimens. Each regimen was administered in three iterations within a week (two days apart), with a minimum one-week washout period before the next anesthesia regimen was tested. On each day of testing, each of six sites per animal was evaluated in four

successive testing rounds using Laser Doppler, Minolta Chroma Meter, and Evaporimeter instruments. A single reading was taken during each round using Laser Doppler and the Evaporimeter. With the Minolta Chroma Meter, four replicate readings were taken and averaged to give one reading for each round. In week one, two animals were dosed starting on Wednesday. In week 1a, during the week washout period for the first two animals, two other animals were dosed on Monday and two on Tuesday. This schedule was repeated until all treatments had been used in each animal. Anesthetized animals were evaluated every other day for 5 days.

Room temperature and humidity were recorded during the performance of all bioengineering methodologies and clinical observations. A digital printing hygrometer/thermometer (Thermo-Hygro Recorder Model NEW 11-661-1 7A, Curtis Matheson Scientific, Inc., Morris Plains, NJ) was used. During data collection, the same room was used throughout Phase III with the hygrometer/thermometer probe placed near the work area. Since room air changes over time was observed to affect the readings of the evaporimeter a plastic tent was constructed and placed over the animals during data collection as described below. The following instruments were used in the order designated below for bioengineering evaluations:

1. Minolta Chroma Meter Model CR-300 evaluated erythema.

2. The Evaporimeter tested the barrier function of the stratum corneum by measuring transepidermal water loss. Due to the nature of this instrument and the need for a controlled environment during its use, the animal was placed in a three sided "tent". The tent was made of 4-in pvc pipe wrapped on three sides with clear plastic and was large enough to allow ample room to work.

3. Laser Doppler perfusion imaging was used as a measure of microcirculation.

Table1. Cross-over Design for Anesthetic Regimens

Animal	Week 1 Anesthesia	Week 1a Anesthesia	Week 2 Anesthesia	Week 2a Anesthesia	Week 3 Anesthesia	Week 3a Anesthesia	Week 6 Anesthesia	Week 6a Anesthesia
1	X	NA[b]	Y	NA[b]	W	NA[b]	NA[b]	NA[b]
2	X	NA[b]	Y	NA[b]	W	NA[b]	NA[b]	NA[b]
3	NA[b]	Y	NA[b]	X	NA[b]	W	NA[b]	NA[b]
4	NA[b]	Y	NA[b]	X	NA[b]	W	NA[b]	NA[b]
5	NA[b]	Z[a]	NA[b]	Y	NA[b]	X	NA[b]	W
6	NA[b]	Z[a]	NA[b]	Y	NA[b]	X	NA[b]	W

[a] Anesthetic regimen Z evaluation was stopped after the first week and replaced by regimen W.

[b] NA = Not Applicable

The day before templates were placed, the ventral abdominal area of each animal was clipped and hair stubble removed with a chemical depilatory compound, Magic® (Carson Products, Co., Savannah, GA). Three 3 x 3 cm sites were drawn approximately 1.5 cm lateral to the teat line on each side of the abdomen of each animal using a plastic template and a marking pen. SM was not applied. Site evaluations generally began 5-10 min after anesthesia was administered, depending upon when the anesthetic regimen used. Evaluations on each animal generally were completed within a 2-hr period, except for the isoflurane inhalation only procedure. This procedure extended beyond 2 hr and this anesthetic regimen was discontinued after the first two animals. The beginning and end times were recorded for each evaluation in each round. Four rounds of readings were conducted with each instrument per animal per evaluation day. Descriptions of the instruments are found in protocol 108, and uses for these instruments are discussed in the references.[5-32] Overviews of high-frequency ultrasound, reflectance colorimetry, evaporimetry, and laser Doppler flowmetry/perfusion imaging can be found in Jorgen Serup's *Handbook of Non-Invasive Methods and the Skin* (CRC Press, 1995).[5] Statistical methods used are reported in the Phase III, Part B, Statistical Report in Attachment F.

3.3 Part C, Development of Full-Skin-Thickness and Superficial Dermal Sulfur Mustard-Induced Burns by Varying Exposure Time

Twenty-five, female weanling Yorkshire or Yorkshire cross pigs, with six ventral sites per pig, were used in Part C of Phase III. Table I-4 in Attachment I identifies animals used in Part C. Six animals were used in a pilot study to examine the depth of burns induced by various times of exposure to 400 µL SM applied percutaneously. The remaining animals then were used to evaluate lesions created by the two selected exposure times. These exposures were accomplished using dosing templates and the procedures described in Summary Report 1 (Attachment C) with the changes described below. Attachment L presents photographs of lesions and histopathology from a representative animal of each exposure group (Millipore water control, 2 min, and 30 min exposure).

3.3.1 Methods

Animal preparation: The day before dosing, animals were weighed and coarse hair removed from the dose area by clipping and application of a chemical depilatory. The chemical depilatory

compound was changed to Magic® shaving powder to be consistent with procedures and products used at USAMRICD. A paste of Magic® shaving powder was prepared and spread over the dose site as instructed by Mr. John Graham and Ms. Patty Matterson of USAMRICD. The powder was mixed with water to form a paste and applied for seven to ten minutes, and then removed with warm, moist gauze. Anesthetic regimen X (Telazol®/Xylazine) was used to induce anesthesia for dosing and for evaluating the lesions. An approximate dosage of 0.044 mL/kg of Telazol® reconstituted with 5 mL of 100 mg/mL xylazine solution was administered im. Injections of this mixture were repeated as needed. The dose and times of anesthetic administration were recorded.

Analgesia: Previous experience showed that weanling pigs cutaneously exposed to SM for 2 hr display some signs of discomfort (e.g., attempts to scratch at the exposure sites), which may be correlated with the degree of erythema and the amount of edema present at these sites starting ~4 hr after exposure. Pain and severe itching sensations occur in humans during the development of cutaneous SM lesions.[1, 33-36] The analgesic buprenorphine (.03 mg/mL Reckitt and Colman Pharmaceuticals, Inc., Richmond, VA) was administered at 0.005-0.01 mg/kg im on a routine basis. The analgesic was administered immediately after exposure and again early the following morning. No analgesic was necessary by day 2. Antibiotics and anti-inflammatory compounds were not used during this phase of the task since they would interfere with the SM reaction and alter the results of the study.

SM Dosing and Decontamination: The 400 µL of undiluted SM was administered topically using a 1000-µL Eppendorf pipette (VWR, Buffalo Grove, IL). Six animals were used for each depth of burn (full-skin-thickness and superficial dermal) with seven pigs in a control group (total of 19 animals). The exposure time identified in the pilot study that produced either a full-skin-thickness or superficial dermal burn at 48 hr post-exposure was applied at all sites. The exposure times selected were expected to produce the specified depth of burn in 80 percent of the animals. After the exposure, the animal was decontaminated by gently dabbing the dose site with a dry sports towel for 30 sec. The decontamination procedure for SM exposure was changed to the dry dab after the Study Director and Lead Technician received an electronic mail message from Mr. John Graham, dated May 6, 1999, requesting the change. Mr. Graham stated that use of water during decontamination appeared to exacerbate the injury in the weanling swine TSP model.

A Miniature Automatic Continuous Air Monitoring System (MINICAMS™) was utilized for verification of decontamination before animals were removed from the fume hood. The MINICAMS™ is a real-time, on-line data acquisition system that employs a solid-sorbent tube to pre-concentrate the agent vapor, a capillary gas chromatographic (GC) column for separation, a flame-photometric detector (FPD), and a personal computer for data acquisition. The MINICAMS™ is designed primarily for the rapid determination of the 8-hr, time-weighted-average (TWA) concentration of chemical-warfare (CW) agents and simulants. Verification of decontamination was performed as outlined in the protocol and as approved by the MREF Environmental, Safety and Health Officer.

A plastic bag covering all of the dosing sites was securely taped to the skin of the animal. After the bag had been attached for a minimum of 15 min, an air sample from the bag was collected for 1 min and analyzed using a method developed for the MINICAMS™ at the MREF. At least five different calibration levels were used for the regression analysis. A check sample, which is a sample of SM concentration ~0.5 TWA was used to verify the instrument's response before and after air samples were acquired.

Measurements/Observations: The following measurements were obtained in the order presented.

1. Minolta Chroma Meter Model CR-300 evaluated the degree of erythema.
2. The Evaporimeter tested the barrier function of the stratum corneum by measuring transepidermal water loss.
3. Laser Doppler perfusion imaging was used as a non-invasive test of the microcirculation.
4. High-frequency ultrasound was used.
5. Clinical observations were recorded.
6. Photographs of lesions were taken.
7. Clinical pathology samples were obtained.

Descriptions and explanation of evaluations and observations are located in MREF protocol 108, amendment 10 in Attachment A. Left side versus right side bias was not considered in this study. Anterior to posterior rotations were only performed on the pilot test

animals as all sites were dosed and exposure times maintained the same per animal in the study. Evaluations were made prior to SM exposure and on day 2 (study day 0 was the day of SM exposure) prior to euthanasia of both pilot and study animals. Animals were euthanatized as previously described in Phase I, Section 3.1.7 of the Phase I Section.

Clinical Observations and Gross Photography: Clinical observations were conducted on study day 2 using the grading scheme presented on page 86 of Amendment 10, Protocol 108. Photography of lesions was conducted along with the clinical observations. The camera was angled such that it was perpendicular to the dermal surface, and at approximately the same distance from the sites. Additionally, pictures were taken of each side at an angle such that the degree of edema (height) could be visualized. Digitized photographs are saved on a CD disk. These photographs allow for future morphological measurements via image analysis, if needed.

Clinical Pathology: Blood samples were collected just prior to agent exposure, and on day 2 just prior to euthanasia. Eight to 10 mL of blood were taken after anesthesia was induced by venipuncture of the anterior vena cava. Blood samples were divided between EDTA tubes and serum separation tubes. The clinical pathology parameters measured were: complete blood cell count (CBC), differential white cell counts, red blood cell (RBC)counts, hematocrit (hct), hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCHb), mean corpuscular hemoglobin concentration (MCHC), electrolytes (chloride, sodium, potassium, calcium, phosphorus), blood urea nitrogen (BUN), creatinine, BUN:creatinine ratio, glucose (hexokinase), alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatine phosphokinase (CPK), amylase, total protein (TP), albumin, globulin and albumin:globulin ratio. Isoenzymes of LDH and CPK were measured on fresh serum samples using classic electrophoretic techniques. Each pre-exposure sample served as the animal's own control reference sample. Each parameter was evaluated for significant changes using appropriate statistical methods as described in the Statistical Report for Phase III Part C in Attachment F.

Histopathology: Skin samples were excised after euthanasia. Each entire exposure site with surrounding normal tissue (full-skin-thickness, and extending ventrally to include the teat line and dorsally to include several cm of normal tissue beyond the lesion) was excised using a scalpel blade. "Full-skin-thickness" was defined as including the panniculus muscle.[2] Where

necessary, deep muscle groups and subcutaneous tissue were trimmed (e.g., external abdominal oblique, latissimus dorsi or serratus anterior).

Sampling methods were demonstrated by Mr. John Graham and Ms. Tracy Hamilton. Each lesion was bisected parallel to the ventral midline. The half closest to the ventral midline was stapled onto an index card to prevent curling, and placed into 10 percent neutral buffered formalin.

The skin sample half closest to the dorsal midline was cut into strips in a direction perpendicular to the midline. Each trimmed piece was ~3-4 mm wide and contained ~2-3 mm of untreated skin and ~10-12 mm of SM-damaged skin. Five such pieces were placed on a piece of gauze and snap frozen using liquid refrigerant (Genetron®22, AlliedSignal, Inc, Morristown, NJ) as demonstrated by Ms. Tracy Hamilton. Following the snap freezing, the tissue samples were wrapped in a double layer of dry-ice cooled aluminum foil, and stored at approximately –70 C until processed. Several strips of untreated ventral skin were collected from each pig and processed similarly.

Frozen samples and formalin-fixed samples were taken to Battelle's Pathology Section for further processing. Frozen sections were cut at ~12 microns (one section from each dosing site). On frozen tissues, the reduction of nitro blue tetrazolium chloride (NBTC) by the cell-bound enzyme nicotinamide adenine dinucleotide diaphorase (NADH-diaphorase) leads to an intense blue, granular precipitate (diformazan granules) at the site of NADH-diaphorase activity. NADH-diaphorase activity has been demonstrated to subside upon cell death, thus viable (blue) and damaged (unstained) cells can be clearly differentiated.[41,43] NBTC staining was used to help determine the depth of the burn. Directions/training for tissue cryopreservation, frozen sectioning on a cryostat, and NBTC staining was provided by MRICD personnel. Over time, stains on frozen tissue can fade, hence NBTC-stained sections were photographed as the stains were generated. For each section, color Kodachromes® (2x2") were prepared which incorporated the junction between normal and SM-damaged skin. Additionally, slides of the center of the SM lesion itself were prepared. Several magnifications were used.

After adequate fixation, formalin-fixed tissue samples were trimmed perpendicular to the ventral midline such that some surrounding normal skin was included in the section. Each trimmed piece measured ~3 cm in length (with ~1 cm of untreated skin and ~2 cm of SM-damaged skin). An additional, shorter piece of full-thickness skin measuring ~1.5 cm long was

trimmed from the untreated area lateral to the treatment site (parallel to the ventral midline and adjacent to the teat line). Both pieces were placed in the same tissue-processing cassette, processed and embedded in paraffin with the epidermal surfaces of both pieces oriented in the same direction. Tissues were stained with hematoxylin and eosin (H&E) for routine histopathologic evaluation. Sections were examined by light microscopy. H&E-stained preparations were examined for depth of necrosis, basal cell necrosis, ulceration, vascular necrosis, hemorrhage, granulation tissue development, and re-epithelialization, and scored according to the following criteria

Morphologic Criteria for Grading Treatment-Related Changes

Depth of necrosis: Defined as the level at which epidermal/dermal structures are within normal limits; grade 0 = entire section is normal; grade 1 = upper epidermis (only) is affected; grade 2 = entire epidermis is affected or missing; grade 3 = entire epidermis affected/missing plus dermis is affected; grade 4 = entire section is affected or structures are missing down to subcuticular-muscle junction. Depth of injury does not imply ulceration *per se*.

Basal cell necrosis, vascular necrosis: Basal epithelial cell or vascular structure (endothelium, vascular wall) necrosis, as evidenced by shrunken, eosinophilic cytoplasm/cell bodies and pyknotic nuclei; grade 0 = normal cells; grade 1 = more than 3 affected cells up to 10 percent of potential area is affected; grade 2 = 10 percent to 25 percent of potential area is affected; grade 3 = 26-50 percent of potential area is affected and grade 4 = greater than 50 percent of area is affected.

Ulceration: Grade 0 = no ulceration seen on section; grade 1 = small focal area of epidermis ulcerated or missing; grade 2 = multifocal lesion, or focal lesion of about 25 percent or less of the epidermal surface; grade 3 = 26-50 percent of epidermal surface affected; grade 4 = greater than 50 percent of surface is affected.

Hemorrhage: Grade 0 = no hemorrhage noted; grade 1 = a few extravasated erythrocytes noted around a few blood vessels within the dermis; grade 2 = clearly discernible blood pooling in up to 25 percent of the dermal area; grade 3 = 26 to 50 percent of dermis is hemorrhagic; grade 4 = greater than 50 percent of dermis is affected.

Granulation: Defined as fibroplasia, with variable influx of macrophages and other inflammatory cells, in the dermis as a result of prior injury. None was seen in these sections.

Re-epithelialization: Defined as a hyperplastic epithelial cell response to prior ulcerating injury. Hyperplastic cells tend to be hyperchromatic and often squamous in appearance as they cover denuded area. None was seen in these sections.

At the end of the experiment, formalin-fixed wet tissues, paraffin blocks and frozen tissue were shipped to USAMRICD for possible special staining and immunohistochemistry. The photographs, and H&E- and NBTC-stained sections were sent to USAMRICD for possible image analysis. In addition, sections from kidney and the diaphragmatic lobe of the lung were collected with half of the sections placed in formalin and half of the sections placed in a fixative supplied by USAMRICD. The kidney and lung samples were sent to USAMRICD for evaluation, and the results are presented in Attachment K.

The histopathological evaluations of H&E- and NBTC-stained sections were conducted by a board-certified veterinary pathologist at Battelle. The results of evaluations of H&E- and NBTC-stained sections were combined for each exposure site, and the overall "depth of burn" characterized for each SM exposure time. Where possible, lesions were classified as either "superficial", "superficial dermal (partial thickness)", "deep dermal (partial thickness)" or "full thickness". Criteria for these burn depths can be found in "Principles and Practice of Burns Management" (Churchill Livingstone, 1996).[2]

Pilot Study: Table J-1 in Attachment J presents the exposure times for each animal in the Pilot Study. Attachment J contains information and data used to determine exposure time for the

Phase III, Part C study. Repeated exposure times were rotated anterior to posterior to prevent an anterior to posterior response bias. Skin samples from the first four animals were evaluated prior to dosing the next two animals. Histopathology determined the depth and severity of lesions at each site and identified exposure times that induced the depth of lesions desired.

Part C Study: Twelve animals were equally divided between the two depth of wound groups (full-skin-thickness and superficial dermal burns) and seven animals were treated exactly the same as the above animals, except Millipore® water was applied to templates. Sham control animals were exposed to the same procedures without SM for the maximum exposure time of 30 min. Animal preparation, evaluation, and data collection were the same as described in the paragraph above.

Six sites (A-F) on the ventral abdomen and two "offsite" control sites (C_1 and C_2), adjacent to sites C and D, were used on each animal. Sites A-F were exposed to SM or Millipore® water according to the group assignment of each animal. These were 1) a 30 min exposure to 400 µl SM to produce a full-skin-thickness wound, 2) a 2 min exposure to 400 µl SM to produce a partial-skin-thickness wound, or 3) Millipore® water control. Sites C_1 and C_2 were not exposed to SM or saline. Wounds were evaluated by clinical observations, photography, histopathology, high frequency ultrasound, reflectance colorimetry (Minolta Chroma Meter), Laser Doppler perfusion imaging, and Evaporimeter data. Each site was evaluated on study day 0 prior to exposure and again on study day 2. Tissue samples for histopathology were collected on study day 2. Replicate readings from the Chroma Meter and Evaporimeter instruments were averaged prior to statistical analysis.

3.3.2. Statistical Methods

Attachment F presents the statistical methods and results for Part C in the Phase III Part C Statistical Reports dated August 10, 2000 and November 2, 2000. The difference between baseline (day 0) and post-exposure (day 2) readings at each site for each instrument was calculated as the primary endpoint for statistical analysis. In addition, the normalized Laser Doppler reading was calculated for each site (A-F) on day 2, as the ratio of the reading for the site divided by the average of the two offsite control readings on the same animal. The normalized Laser Doppler readings are presented in the results section of this report. For this

instrument, a large variation was observed in the differences between readings in day 0 and day 2. Normalizing the readings to a control site reading taken on the same day provided a more stable response. Analysis of variance (ANOVA) models were fitted to the Ultrasound, Chroma Meter, Laser Doppler, and Evaporimeter data from control sites to determine (1) whether there was a positional effect and (2) whether there was a systemic effect due to SM exposure. Additional ANOVA models were fitted to the data from sites A-F to assess the effects of SM exposure using these instruments.

In all, three models were fitted to the readings from each instrument:

- **Model 1** included readings from sites A-F, C_1 and C_2 on control animals and sites C_1 and C_2 on full-and partial-skin-thickness animals to test for positional effects. Model 1 was formulated as follows:

  (1) $\textbf{Response}_{ij} = \mu + \alpha_i + \beta_j + \varepsilon_{ij}$

  where Response_{ij} = difference in instrument readings from day 0 to day 2 for the i^{th} site on the j^{th} animal

  μ = overall average value of the response

  α_i = effect of i^{th} site

  β_j = random effect of j^{th} animal

  ε_{ij} = random variation for the i^{th} site on the j^{th} animal

- **Model 2** included readings from C_1 and C_2 control sites on all animals to test for systemic effects of SM-exposure on the control sites. Model 2 was not fitted to the normalized Laser Doppler readings because the C_1 and C_2 sites are normalized to themselves. Model 2 was formulated as follows:

  (2) $\textbf{Response}_{ij} = \mu + \alpha_i + \beta_j + \varepsilon_{ij}$

  where Response_{ij} = difference in instrument readings from day 0 to day 2 for the i^{th} treatment on the j^{th} animal

  μ = overall average value of the response

  α_i = effect of i^{th} treatment

  β_j = random effect of j^{th} animal

  ε_{ij} = random variation for the i^{th} site on the j^{th} animal

- **Model 3** included readings from sites A-F of all animals to assess the effects of SM-exposure. Model 3 was formulated as follows:

(3) $\textbf{Response}_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \delta_k + \varepsilon_{ijk}$

where Response_{ijk} = difference in instrument readings from day 0 to day 2 for the i^{th} site with the j^{th} treatment on the k^{th} animal

μ = overall average value of the response

α_i = effect of i^{th} site

β_j = effect of j^{th} treatment

$\alpha\beta_{ij}$ = interaction effect between i^{th} site and j^{th} treatment

δ_k = random effect of k^{th} animal

ε_{ijk} = random variation for the i^{th} site with j^{th} treatment on the k^{th} animal

Each model was fitted using the SAS (V8) MIXED (Cary, NC) procedure. For Models 1 and 3, statistical contrasts of the model parameters were used to evaluate positional effects including front-to-back and onsite vs. offsite (Model 1). Bonferroni adjustments were made to maintain an overall 0.05 level of significance over the multiple comparisons within each model. For Models 2 and 3, the Tukey-Kramer multiple comparisons procedure was used to evaluate treatment effects. In addition, model 3 was also fitted to the depth of burn continuous variable in the histopathology data set, and to the edema length, edema breadth, edema height, edema area, wound length, wound width and wound area variables in the clinical observation data set. Models 1 and 2 could not be fitted for these endpoints, as all readings were zero at the control sites on both day 0 and day 2.

Wound size measurements were calculated as described (elliptical) in section 3.4 Analyses of Task 94-33 Phase I Model Development. The rest of the histopathology and clinical observation data were categorical, of which there were two types: binary (0 or 1) variables indicating the absence or presence of a certain histopathologic or clinical observation endpoint, and endpoints scored on a 0 to 4 severity scale. Binary endpoints included ulceration and hemorrhage in the histopathology data set, and the hemorrhage purple endpoint in the clinical observation data set. The histopathologic endpoints scored 0-4 were basal cell necrosis, depth of necrosis, and vascular necrosis, while the clinical observation endpoints were erythema extent, erythema description, edema, and necrosis.

For the binary histopathology and clinical observation data, Fisher's Exact tests were calculated to compare treatments. For the endpoints with severity scores, General ANOVA Scores tests were computed to compare treatments. There were endpoints in both the histopathology and clinical observation data where all values were zero and could not be analyzed. These data consisted of the granulation and re-epithelization histopathologic endpoints, and the exudate, eschar, eschar scab percent covered, and infection clinical observation endpoints.

Both Pearson's and Spearman's correlation coefficients were calculated to examine the relationship between normalized Laser Doppler Readings and wound depth (mm) and depth of necrosis (scored as 0-4). The SAS (V8) CORR procedure was used for this analysis. The full and partial groups were included in the analysis.

4.0 RESULTS

4.1 Part A, Solubility, Stability, and Hydrolysis Testing

Preliminary results to determine a chromatographable solvent showed that the four liquids [peanut oil, propylene glycol, polyethylene glycol (PEG) 200 and PEG 400] being evaluated were not very soluble in hexane, but more soluble in chloroform. The solubility results indicated that methylene chloride would be the best solvent both for diluting the samples and for the GC analysis. GC results of the four liquids by themselves demonstrated that they would not interfere with the SM analysis via GC-FID. Figures 1 to 4 in the Internal Chemistry Report dated March 1999 in Attachment G show chromatograms for each of the liquids. Although there are several components observed in these chromatograms, they are outside of the SM Retention Time of 5.65 min (see Figures 5 and 6 in the Internal Chemistry Report). PEG 400 was not soluble in hexane and the least soluble in chloroform, so it was not included in the subsequent miscibility and stability testing of SM. Peanut oil and PEG 200 were found to be miscible with neat SM but propylene glycol was not. The propylene glycol/SM mixture was cloudy and separated into layers after a few minutes. Consequently, the propylene glycol-SM mixture could not be diluted for stability testing. The stability results for the SM/peanut oil and

SM/PEG 200 mixtures are presented in Table 2. Chromatograms of the samples after approximately 19 hr are shown in Figures 5 and 6 of the Internal Chemistry Report in Attachment G.

TABLE 2. ANALYSIS RESULTS FOR STABILITY SAMPLES

Sample Description	Expected SM Conc.	Measured SM Conc.	Percent of Expected
50% SM/Peanut oil, Time 0, Injection 1	1.16 mg/ mL	1.17 mg/ mL	101 %
50% SM/Peanut oil, Time 0, Injection 2	1.16 mg/ mL	1.17 mg/ mL	101 %
50% SM/Peanut oil, Time 0, Injection 3	1.16 mg/ mL	1.15 mg/ mL	99 %
50% SM/PEG 200, Time 0, Injection 1	1.16 mg/ mL	1.17 mg/ mL	101 %
50% SM/PEG 200, Time 0, Injection 2	1.16 mg/ mL	1.15 mg/ mL	99 %
50% SM/PEG 200, Time 0, Injection 3	1.16 mg/ mL	1.18 mg/ mL	102 %
50% SM/Peanut oil, ~19 hr, Injection 1	1.16 mg/ mL	1.19 mg/ mL	103 %
50% SM/Peanut oil, ~19 hr, Injection 2	1.16 mg/ mL	1.15 mg/ mL	99%
50% SM/Peanut oil, ~19 hr, Injection 3	1.16 mg/ mL	1.16 mg/ mL	100 %
50% SM/PEG 200, ~19 hr, Injection 1	1.16 mg/ mL	1.15 mg/ mL	99%
50% SM/PEG 200, ~19 hr, Injection 2	1.16 mg/ mL	1.18 mg/ mL	102 %
50% SM/PEG 200, ~19 hr, Injection 3	1.16 mg/ mL	1.17 mg/ mL	101 %

The results for the SM/peanut oil and SM/PEG 200 mixtures stored in a freezer at approximately –70° C are presented in Figures 1 to 6 of the Internal Chemistry Report in Attachment G. The overall average values, standard deviations (STD) and relative standard deviations (RSTD) for the mixtures stored in the freezer are shown in Table 3. The average values from Table 3 are plotted in Figures 1 to 6 in Attachment G, and the limits shown in these figures are based on the standard deviation values presented in Table 3 (Upper Limit = Average + 1 STD; Lower Limit = Average - 1 STD). The results for the eight-day stability test of mixtures stored at room temperature are given in Table 4.

TABLE 3. STATISTICAL RESULTS FOR THE STABILITY MIXTURES STORED IN A FREEZER AT APPROXIMATELY –70 C FOR THIRTY-FIVE DAYS

Sample	Average Percent of Expected	Standard Deviation	RSTD in Percent
25% Peanut Oil	99%	0.073	7.38%
25% PEG 200	100%	0.065	6.53%
50% Peanut Oil	102%	0.043	4.20%
50% PEG 200	97%	0.027	2.78%
75% Peanut Oil	93%	0.057	6.06%
75% PEG 200	91%	0.057	6.29%

TABLE 4. ANALYSIS RESULTS FOR THE STABILITY MIXTURES STORED AT ROOM TEMPERATURE FOR EIGHT DAYS

Sample	Average Percent of Expected
25% Peanut Oil	93%
25% PEG 200	108%
50% Peanut Oil	98%
50% PEG 200	99%
75% Peanut Oil	94%
75% PEG 200	92%

Dose Confirmation: Dose confirmation samples were analyzed via GC-FPD, like the stability samples, except the GC injector temperature was 250 C instead of the lower temperature used for stability samples containing either peanut oil or PEG. The instrumental parameters used for dose confirmation samples are listed in Table 1 of the Internal Chemistry Reports in Attachment G. The linearity of the analysis method was determined by analyzing at least four calibration standards. All of the measured SM concentration values for dose confirmation samples collected during this task were within 10 percent of the expected value except for four samples that were analyzed in February and March of 1997. The concentrations for these samples were between 87 and 89 percent of the expected values. The lesions created with the SM used during this time period were confirmed as full-skin-thickness burns by histopathology.

Decontamination: In Phase III, the decontamination procedure was changed. Mr. John S. Graham reported in an e-mail dated May 6, 1999 that water decontamination appeared to exacerbate the lesion when used during TSP screening in the weanling pig model. Mr. Graham conducted a pilot study to determine if using or not using the water wipes would increase the SM off-gassing times (prolong the animals stay within the chemical fume hood). There was no statistically significant difference in the off-gassing times between the pilot animals and historical data (animals decontaminated with water). The decontamination procedure for Phase III animals and future studies is to perform a dry pat of the area for 30 sec, recover the animal from anesthesia and hold the animal in a chemical fume hood (within a modified transport crate) overnight for off-gassing.

4.2 Part B, Anesthesia Effects

Problems encountered during Part B of Phase III, as assessed by technicians, were as follows. Anesthesia regimen Z (Isoflurane only) was difficult to test within the 2-hr time constraint due to the techniques involved in initiating anesthesia. Manual restraint of the animal while administering inhalation anesthetic by mask was required for ~45 min before anesthesia was induced. Biomechanical instrumentation evaluation was prolonged beyond the 2-hr time constraint. Regimen W (Ketamine/Xylazine) required too many injections during the 2-hr period. Regimen Y (Telazol/Xylazine/ISO) required repeated intubation of the animal on each test day, and usually, after the second test day, the animal's larynx was edematous. Repeated intubations were difficult and the animal had difficulty breathing after removal of the endotracheal tube.

The Phase III, Part B Statistical Report dated June 15, 2000 is in Attachment F. The statistical models estimated the responses for the Laser Doppler, Evaporimeter, and Chroma Meter by anesthesia, iteration, and round. These results are in Table 3 of the Statistical Report in Attachment F, and in Figures 1 through 3. Table 4 and Figure 4 of Attachment F show the estimated response when anesthesia X, Y, or W were administered. These tables and figures demonstrate that:

- For Laser Doppler readings, anesthesia W yielded the highest response and anesthesia Y yielded the lowest response. As indicated in Table 4 and Figure 4 of the Phase III Part B Statistical Report in Attachment F, the mean responses were significantly different for all three anesthesia regimens. The first sampling round had the highest response of each sampling iteration. Mean response declined on later study days for regimens X and W, but not for Y (Figure 1 in Attachment F).

- For Evaporimeter readings, anesthesia W yielded the lowest response on average and anesthesia X and Y yielded higher responses (Table 4 and Figure 4 in Attachment F). The first sampling round had the highest response and the fourth sampling round had the lowest response. Mean responses declined over three days of experiments for each anesthesia (Figure 2 of Attachment F).

- For Chroma Meter readings, anesthesia W yielded the lowest response and anesthesia X and Y yielded higher responses, which were not significantly different from each other on average (Table 4 and Figure 4 in Attachment F). The first sampling round had the highest response for all three regimens. Regimens X and W had consistent readings over 3 days while regimen Y had greater day-to-day variability (Figure 3 in Attachment F).

4.3 Part C, Development of Full-Skin-Thickness and Superficial Dermal Sulfur Mustard-Induced Burns by Varying the Exposure Time in Weanling Swine

The Phase III Statistical Reports for Part C are found in Attachment F. Problems encountered during Part C of Phase III, as assessed by technicians, were as follows. The physical anatomy (e.g., a broad and/or deep thoracic area, prominent ribs, curvature of rib cage and abdominal area) of certain animals interfered with the creation of a well-defined lesion, particularly in the anterior area. In addition, accurate ultrasound readings were difficult on some animals.

4.3.1. Pilot Study

Tables of histopathologic scoring for each site of each pilot animal are in Attachment K. Nitroblue tetrazolium chloride–stained frozen sections were used to assess the depth of a burn for

a 30-min exposure at ~0.65 mm on average. Depth of necrosis was graded at a maximum score of 4 at this time point. Laser Doppler (LDPI) analyses, using a calculated ratio of lesion flux to background flux, did not indicate any significant decrease (observable) in blood flux ratio between the 30-min, 40-min, and 60-min SM exposures. LDPI results from two animals with at least two sites with the same exposure time were 30 min = 0.43, 40 min = 0.58, 60 min = 0.63, and 60 min = 0.44. Histopathologic scoring of hemorrhage and vascular damage indicated no difference between 30 and 60 min exposure times. Thus, 30 min was selected as the exposure time for a full-skin-thickness lesion.

Two animals (with at least one site per animal except where indicated, n=2 sites total) were used to determine the superficial dermal (partial-skin-thickness) SM burn. Nitroblue tetrazolium chloride–stained frozen sections assessed the depth of burn at approximately 0.005 mm on average at 1 min, 0.12 mm at 2 min, 0.33 mm at 3 min, 0.30 at 4 min (n=1), and 0.27 mm at 5 min (n=3). Histopathologic scoring for depth of necrosis (maximum score of 4) averaged 1.5 at 1 min, 2 at 2 min, 2.5 at 3 min, 2.5 at 4 min and 2.7 at 5 min. Histopathologic scoring for necrosis of basal cell epithelium (maximum score of 4) was 1 at 1 min, 2 at 2 min, 3 at 3 min, 3.5 at 4 min, and 4 at 5 min. A 2-min cutaneous exposure to 400 μL SM was selected to produce a partial-skin-thickness burn since the basal cell score was greater for the 2 min than 1 min and the depth of burn was deeper for the 2 min than 1 min. There was not a significant difference in depth of burn or depth of necrosis score for exposure times between 2 min and times greater than 2 min. Necrosis of basal cell epithelium showed numerical increase as exposure time increased above 2 min. Two-min lesions were more consistent and uniform within the lesion and between other 2 min lesions than those produced using times < 2 min.

4.3.2. Phase III Part C Study

Descriptive statistics for ultrasound, Chroma Meter, Laser Doppler, Evaporimeter, and burn depth data are provided in Table A-1, Appendix A of the Phase III, Part C Statistical Report in Attachment F. Tables 1 through 4 of the same report present model-predicted means, standard errors, and hypothesis test results for the ultrasound, Chroma Meter, Laser Doppler, and Evaporimeter data.

Table 1 of the Phase III, Part C Statistical Report dated August 10, 2000 in Attachment F also provides the results of Model 1 evaluations of positional effects for control sites (C_1 and C_2) from each animal on study and all sites (A-F, C_1 and C_2) from control animals. Table 1 of the Phase III, Part C Statistical Report on additional analysis of Laser Doppler data dated November 2, 2000 provides analogous information for the normalized Laser Doppler endpoints. The third and fourth columns of these tables contain the model-estimated mean and standard error (SE) for each of the continuous parameters. Almost all parameters showed a reduction between day 0 and day 2. This phenomenon is normal for control animals (multiple anesthetic injections) and was noted previously in the Phase III, Part B anesthesia experiments. The last five columns contain the p-values for statistical comparisons evaluating front-to-back and onsite vs. offsite position effects. The normalized Laser Doppler readings showed evidence of an onsite-to-offsite positional effect in control animals. The model estimated mean normalized Laser Doppler reading for sites ABCDEF was 1.3 times greater than sites C1 and C2. As there were four contrasts performed for this model, p-values were compared at an alpha of 0.05/4=0.0125, using the Bonferroni adjustment for multiple comparisons.

Table 2 of the Phase III, Part C Statistical Report dated August 10, 2000 in Attachment F presents the results from Model 2 comparisons that were performed to determine whether there was an effect of SM-exposure on the control sites of the three treatment groups. The third and fourth columns of this table contain the model-estimated means and SEs for each of the four parameters. The last three columns contain the unadjusted p-values and the Tukey adjusted p-values for differences between treatments. When considering significance, an alpha of 0.05 was used to compare to the Tukey adjusted p-values. No significant differences were found, indicating no systemic effects due to either exposure time were seen for the offsite controls, C1 and C2.

Table 3 of the Statistics Reports dated August 10, 2000 and November 2, 2000 (normalized Laser Doppler readings only) presents the results of Model 3 evaluation of SM effects on treated sites (A-F) on all animals. Ultrasound readings had a significantly greater change in the full-skin-thickness (30 min) group than the control or partial-skin-thickness (2 min) group, which did not differ significantly from each other. Chroma Meter and Evaporimeter recording changes were significantly greater than those of the control group for both the partial-skin-thickness and full-skin-thickness groups. The mean Chroma Meter and

Evaporimeter changes did not differ between the partial-skin-thickness and full-skin-thickness groups. Mean normalized Laser Doppler readings were significantly different from each other for all treatment groups. Group means (SE) were 2.11 (0.08), 1.31 (0.07), and 0.96 (0.08) for the partial thickness, control, and full-thickness groups, respectively.

Burn depth was significantly different between the full-skin-thickness and control group. Burn depths in the partial-skin-thickness group were not statistically different from either the control or the full-skin-thickness group. Edema length, breadth, height, and area were significantly different between the full-skin-thickness and partial-skin-thickness, and between full-skin-thickness and control groups, but the partial-skin-thickness and the control group were not significantly different. Wound length, width, and area showed significant differences between control and partial-skin-thickness, and between control and full-skin-thickness groups. The partial-skin-thickness and full-skin-thickness groups were also significantly different in wound width and area, but not in wound length.

Table 4 of the Phase III, Part C Statistical Reports dated August 10, 2000 and November 2, 2000 (normalized Laser Doppler readings only) in Attachment F presents the results from the Model 3 evaluation of position effects at treated sites (A-F) of all animals within each treatment group. For ultrasound readings, changes at site CD were significantly greater than site AB in the full-skin-thickness group. Chroma Meter changes were significantly greater at site EF than at site AB in the partial-skin-thickness group. Mean normalized Laser Doppler readings decreased significantly from front (sites A and B) to middle (sites C and D) to back (sites E and F) in the partial group. In the full group, the mean for front sites was significantly greater than that of back sites. Although a similar trend was present, comparisons of middle sites to front and back were not statistically significant in the full group. There were no significant position effects for Evaporimeter readings.

There were not any significant position effects within any of the treatment groups for burn depth, wound length, wound width and wound area. However, some significant results were seen in the full-skin-thickness exposure group for the various edema endpoints. For edema area and breadth, site EF was significantly different from both site AB and CD. Edema height was significantly different between sites AB and CD, and edema length was different between sites AB and EF.

Categorical Data Analyses in Clinical Observation and Histopathologic Data: Table 5 of the Phase III, Part C Statistical Report in Attachment F presents the counts and percentages of observations in each category of the histopathologic and clinical observation endpoints. These endpoints are scored as present or absent. This table also includes p-values from Fisher's Exact Tests comparing the three different groups to each other. Results were considered significant when p-values were less than the Bonferroni adjusted alpha of 0.05/3 or 0.0167. Table 6 of the same report presents the number and percentages of animals for each score using the 0-4 severity scale for histopathologic and clinical observation endpoints. The results of the General ANOVA Scores test to compare the control, partial- and full-skin-thickness treatment groups for histopathologic and clinical observation endpoints are also presented in Table 6.

Histopathologic endpoints: As shown in Table 5 of the Phase III, Part C Statistical Report in Attachment F, the incidence of hemorrhage in the control group was significantly less than in the partial- and full-skin-thickness groups, while incidence of hemorrhage was not significantly different between the partial- and full-skin-thickness groups. Incidence of ulceration in the partial-skin-thickness group was significantly greater than in both the control and full-skin-thickness groups, while the control and full-skin-thickness group incidences were not significantly different from each other.

Severity scores for basal cell necrosis, depth of necrosis and vascular necrosis generally increased from the control to partial- to full-skin-thickness exposure groups, and all the treatment groups were significantly different from each other in all three endpoints (Table 6 of the Statistical Report dated July 18, 2000 in Attachment F).

A general histopathologic description of sites for each exposure time follows. Sections of skin taken from pigs exposed to sham agent were essentially normal. Sections exposed to agent for 2 min tended to have epidermal injuries that incompletely (or multifocally) penetrated the full breadth of the application site, and to have injuries that were less deep than sites exposed for 30 min. Two-min sites had irregularly affected overlying epithelium, and were more likely to have purulent inflammation associated with the injured cells. In addition, sites exposed for 2 min were more likely than 30-min sites to have focal erosions/ulcerations. These ulcerations were pin-point focal lesions, and generally did not penetrate much below the basal cells/basement membrane of the epithelium. Sites exposed for 30 min usually had uniformly necrotic epithelium with significant dermal involvement. The overlying epithelial cells were contracted

and eosinophilic. Although the total injury was nearly always more uniform and deeper than that seen in 2-min sites, focal ulceration of the epithelium was uncommon.

Histopathologic evaluation (Veterinary Pathology Report in Attachment J) of the diaphragmatic lobe of the left lung found moderate and diffuse congestion and intralobular and interlobular edema . Intrabronchiolar edema and hemorrhage were thought due to hypostatic congestion and not due to systemic effects of SM. The kidneys were reported as normal.
Clinical observation endpoints: Incidence of hemorrhage in the full-skin-thickness group was significantly greater than that in the control and partial-skin-thickness groups. Purple hemorrhagic areas were not observed in the control or the partial-skin-thickness groups.

As shown in Table 6 of Attachment F, severity scores for edema were significantly different among all of the groups. While severity scores for erythema description and erythema extent in the control group were significantly lower than those in the partial- and full-skin-thickness exposure groups, severity scores in the partial- and full-skin-thickness groups were not significantly different from each other. Necrosis was significantly greater in the full-skin-thickness exposure group than the controls at the Bonferroni adjusted alpha of $0.05/3 = 0.0167$, and the partial- and full-skin-thickness group comparison ($p=0.025$) was marginally significant.
Correlation Analysis: The correlation analysis suggests that normalized Laser Doppler readings on day 2 are significantly correlated with wound depth ($p=0.004$) and depth of necrosis ($p<0.001$). Results of the Pearson correlation analysis are reported for wound depth, while Spearman's is presented for depth of necrosis.

5.0 CONCLUSIONS

5.1 Part A, Solubility, Stability, and Hydrolysis Testing

Propylene glycol can not be used with SM because they do not mix. Since PEG 400 was not soluble in the GC solvent, it was not tested with SM. SM/peanut oil and SM/PEG 200 mixtures at the 25, 50 and 75 percent SM levels are stable for more than 8 days when stored at room temperature and more than 35 days when stored in a freezer at $\geq$ -70 C.

5.2 Part B, Anesthesia Effects

For Laser Doppler readings, the three anesthesia regimens yielded a significantly different response level, but none was preferable. The anesthesia regimens had similar effects within a day, but effects of anesthesia X (Telazol/Xylazine) and W (Ketamine/Xylazine) declined over treatment days while Y (Telazol/Xylazine/ISO) was less consistent in its effect. The depth of anesthesia was not controlled well with anesthesia regimen Y. No regimen was clearly preferable for the Laser Doppler instrument as all regimens affected cutaneous blood flow. The more anesthesia the animal was given during the measuring period, the greater the reduction in cutaneous blood flow. The reduction in cutaneous blood flow over multiple treatment days was particularly evident with the injectable anesthetics. The decline observed over the multiple treatment days for the injectable anesthetics was probably the result of residual anesthetic. Inhalation anesthetics like Isofluorane are eliminated from the body more completely and in less time than injectable anesthetics.

For the Evaporimeter instrument, anesthesia regimen W yielded a lower response than X and Y. There was a decline in response over time within a day that indicated that the Evaporimeter was sensitive to the stage of anesthesia. The response also declined over multiple treatment days for each anesthesia. Regimen X or Y was preferable for Evaporimeter readings.

For the Chroma Meter instrument, anesthesia regimens X and Y yielded a higher response than W, indicating a greater blanching effect for anesthesia W. The anesthetic regimens behaved similarly within a day, with round one yielding a higher response than subsequent rounds indicating that the Chroma Meter was sensitive to the stage of anesthesia. Response over multiple days was less variable for anesthesia X and W than for Y. Either regimen X or W was suitable, with X preferred due to lesser blanching effect.

Regimen X , Telazol®/Xylazine is recommended for use in future experiments as it is the most suitable when readings are taken with all three instruments. It is also recommended that Chroma Meter and Evaporimeter readings be taken first as they are most sensitive to time following induction of anesthesia.

5.3 Part C, Development of Full-Skin-Thickness and Superficial Dermal Sulfur Mustard-Induced Burns by Varying the Exposure Time in Weanling Swine

5.3.1. Pilot Study

An exposure time of 30 min for a full-skin-thickness burn and 2 min for a partial-skin-thickness burn using 400 µL of undiluted SM applied to ventral abdominal sites were selected. Histopathologic endpoints were chosen as the selection criteria for characterizing the burn. Depth of burn in mm, depth of burn severity score, and necrosis of basal epithelium were the key histopathologic endpoints used to determine the exposure times. In the pilot study, necrosis of basal cell epithelium increased in area with exposure times greater than 2 min and was more consistent in the lesion created. A greater emphasis perhaps should have been placed on this criterion in selecting the SM exposure time for partial-skin-thickness burns.

5.3.2 Phase III, Part C Study

Lesions produced by various exposure times (2 and 30 min) to 400 µL undiluted SM or saline applied to ventral abdominal sites were characterized and described using clinical observation endpoints, histopathological endpoints, and non-invasive biomechanical instruments. There was mixed evidence that the control, partial-skin-thickness and full-skin-thickness groups were statistically significantly different from each other when using these methods. The fact that a difference could not always be detected statistically between one or more group is not as important as establishing a historical database for each endpoint and parameter measured. All three groups were significantly different from each other in wound area, wound width, basal cell necrosis, depth of necrosis, vascular necrosis, and edema, which are predominantly histopathological endpoints. Histopathological evaluations and descriptions were key in characterizing the 30-min and the 2-min exposure-time lesions.

Characterization of the predominant endpoints for each exposure time are summarized as follows. The saline-exposed sites were normal for most parameters. One site had a hemorrhage and less than10 percent of the area involved in basal cell necrosis and necrosis of the epidermis. This was an incidental finding (scratch, trauma from handling or moving/lying down in the cage) and not related to the application of saline.

Sites exposed for 30 min to 400 μL of undiluted SM were generally erythematous (moderate to severe with a Chroma Meter mean difference of 8.89 ± SE 0.63), edematous (mean area of 842.91 mm^2, breadth of 32 mm, edema height of 4.3 mm, and edema length of 31 mm), burn depth of 1.36 mm ± SE 0.29, and generally the entire dosed area was affected with some extension beyond the dosed site for most clinical and histopathological endpoints. Necrosis affected the entire epidermis and through the basal cell layer into the dermis in at least 86 percent of the sites. Vascular necrosis affected 10 to 50 percent of the area at 94 percent of the sites. Trans-epidermal water loss was present with a measured difference of 3.73 ± SE 1.57 between day 0 and day 2. Laser Doppler measurement of blood flow flux normalized to offsite controls was reduced (0.96 ± SE 0.08) compared to the control group (1.31 ± SE 0.07). The reduced blood flow resulting from damaged vessels in the affected area for the 30-min exposure time group was significantly different from control animals.

Sites exposed for 2 min to 400 μL of undiluted SM were generally erythematous (pink to deep red, with red being observed predominantly, and a Chroma Meter mean differences of 8.4 ± SE 0.63). Edema was not present at 48 hr. A burn depth of approximately 0.76 mm ± SE 0.29 and generally multifocal areas of damage among normal appearing areas were observed. Necrosis affected the entire epidermis with some extension into the dermis. Necrosis of the basal cell layer in 94 percent of the sites had a severity score grade less than 2, indicating that only 10 to 25 percent of the potential area was affected. Vascular necrosis affected less than 10 percent of the area in 22 percent of the sites, with the remaining sites graded as normal. Trans-epidermal water loss was measured as a difference of 3.73 ± SE 1.57 between day 0 and day 2. Laser Doppler measurement of blood flow flux normalized to offsite controls showed an increase (2.11 ± SE 0.08) compared to control animals (1.31 ± SE 0.07). An increase in blood flow to the affected area was observed at sites in the 2-min exposure time group as compared to the controls and 30-min exposure groups.

A full-skin-thickness SM burn in this swine model was created with 30 min exposure to 400 μL of undiluted SM, but a 1 hr exposure may be preferable in future studies. Histopathology showed that a full-skin-thickness burn was observed over at most 50 percent of the exposed area in 94 percent of the sites (determined by vascular necrosis score and general impression reported by pathologist). If the entire exposed area is to be full-skin-thickness for all exposure sites, then the SM exposure time should be increased above 30 min. The severity of the wound observed on

day 2 with the 30-min exposure is considered a deep dermal to full-skin-thickness burn and in time will become full-skin-thickness. For a partial-skin-thickness burn, the exposure time should be increased to between 5 and 10 min in future studies. The 2-min exposure sites were inconsistent and not uniform within and between animals. Histopathology confirmed that a uniform, consistent partial-skin thickness burn had not been attained with this exposure. Histopathologic data obtained in the pilot study and in study animals predict that a 7-min SM exposure time would provide a consistent partial-skin-thickness burn.

Systemic effects due to SM-exposure were alluded to in Phase I, Part B, and found to be clinically insignificant in earlier experiments conducted under MREF Task 94-33. In Phase III, Part C, no systemic effects of SM exposure were observed at the offsite control sites (C_1 and C_2) using ultrasound, Chroma Meter, Laser Doppler, or Evaporimeter readings. No differences between the onsite and offsite control sites were observed. Therefore, the use of within animal control sites in future experiments utilizing this model and these instruments is recommended.

The biomechanical instruments used in this study were non-invasive and are routinely used in dermatology clinics. Their continued use, particularly in treatment screens and especially for products that pass multiple species screens, is recommended.

5.3.2.a Exposure Time of 30 min

An average burn depth difference from day 0 to day 2 was 1.36 mm for the 30 min SM exposure time. Sites exposed to SM for 30 min usually had uniformly necrotic epithelium with significant dermal involvement. The overlying epithelial cells were contracted and eosinophilic. Even though less than 20 percent of the vascular structures indicated that the area was affected, the pathology indicated that this exposure would become more severe if allowed to develop over time. The 30-min SM exposure time produced a deep dermal to full-skin-thickness lesion. Histopathology: Histopathologic descriptions and data analyses confirmed that a more uniform and consistent lesion was obtained with the 30-min exposure time, than with the 2-min exposure time. For the 30-min exposures, 92 percent of the sites were scored for basal cell epithelium necrosis with a severity grade of 3 (26-50 percent of the area affected). For depth of necrosis (score 0 to 4) for the 30-min exposure, 86 percent of the sites were scored with a severity grade of 4 (over 50 percent of area is affected). For vascular necrosis (score 0 to 4) in the 30-min exposure group, 67 percent of the sites were scored with a severity grade of 1 (more than 3

affected cells up to 10 percent of the potential area affected), 6 percent were graded 0 (normal), and 28 percent were graded 2 (10-25 percent of the potential area affected). Ulceration (absent or present) for this exposure was absent at 97 percent of the sites and present in 3 percent. Hemorrhage (absent or present) was present at 100 percent of the sites.

Clinical Observations: Edema (score 0 to 4) at 58 percent of the sites was scored as 3 (moderate, ~2-3 mm, well defined). Thirty three percent of the sites were scored as 4 (severe, area raised $\geq$ 4 mm and extending beyond lesion border) and 8 percent as 0 (normal). Erythema (score 0 to 3) at 72 percent of the sites was scored as 2 (red), at 17 percent of the sites was scored as 3 (deep red), and at 11 percent of the sites was scored as 1 (pink). Erythema extent (score 0-2) at 78 percent of the sites was scored as 1 (present along border and within border) and at 22 percent was scored as 2 (beyond border) . Extent of necrosis (necrosis, score 0 to 4) at 83 percent of the sites was scored as 0 (none) and at 17 percent of the sites as 2 (at least 25 percent but less than 50 percent of the original dosing area involved). Hemorrhagic purple discoloration was absent at 56 percent of the sites and present at 44 percent of the sites.

Biomechanical Instrumentation Measurements: Ultrasound difference from day 0 to day 2 for the 30-min exposure was a mean of 1.46 ± SE 0.20. Chroma Meter difference for the same time period was 8.89 ± SE 0.63. Evaporimeter difference was a mean of 3.73 ± SE 1.57, but not clinically significant. Mean Laser Doppler readings normalized to offsite controls on day 2 was 0.096 ± SE 0.08.

5.3.2.b Exposure Time of 2 min

Average burn depth difference from day 0 to day 2 was 0.76 mm for the 2-min exposure time. Sites exposed for 2 min were not uniform and appeared to be more multifocal in damage. The 2-min SM exposure time did not produce a consistent and uniform partial-skin-thickness lesion.

Histopathology: Histopathologic descriptions and data analyses for day 2 reported an inconsistent lesion and not a uniform, partial-skin-thickness burn. Necrosis of the basal cell epithelium (score 0 to 4) with the 2-min burn at 67 percent of the sites (n=36) was scored as a 2 (10 to 20 percent of the potential area affected). Twenty eight percent of the sites were scored as 1 (more than 3 affected cells up to 10 percent of potential area affected), and 6 percent of the sites were scored as 3 (26-50 percent of the area affected). Depth of necrosis (score 0 to 4) for

the same exposure time at 100 percent of the sites was scored as 3 (entire epidermis affected/missing and dermis is affected). Vascular necrosis (score 0 to 4) at 78 percent of the sites was scored 0 (normal) and 22 percent were graded as 1 (more than 3 affected cells up to 10 percent of the potential area affected). Ulceration (absent or present) was absent at 75 percent of the sites and present at 25 percent. Hemorrhage (absent or present) was present at 92 percent of the sites.

Clinical Observations: Edema (score 0 to 4) at 81 percent of the sites (n=36) was scored as 0 (none) and at 19 percent of the sites were scored as 1 (minimal, barely perceptible or questionable) for 2-min SM exposure sites. Erythema (score 0 to 3) at 72 percent of the sites was scored as 2 (red), at 19 percent of the sites as 1 (pink), and at 8 percent of the sites as 3 (deep red). Erythema extent (score 0-2) at 75 percent of the sites was scored as 1 (present along border and within border) and at 25 percent as 2 (beyond border and inclusive of number 1). Extent of Necrosis (necrosis, score 0 to 4) at 100 percent of the sites was scored as 0 (none). Hemorrhagic purple discoloration was absent at 100 percent of the sites.

Biomechanical Instrumentation Measurements: Ultrasound difference from day 0 to day 2 for the 2 min SM exposure was a mean of 0.27 ± SE 0.20. Chroma Meter difference for the same time period was 8.40 ± SE 0.63. Mean Laser Doppler readings normalized to offsite controls on day 2 was 2.11 ± SE 0.08. Evaporimeter difference was a mean of 2.45 ± SE 1.57 and not clinically significant.

5.3.2.c Millipore Water-Exposed Control Sites

Control sites for this study were normal, as reported by Battelle's pathologist. Only one site out of 42 was reported with epidermal damage. This epidermal damage probably occurred during the handling of the animal prior to exposure and is not related to the study.

Histopathology: Necrosis of the basal cell epithelium (score 0 to 4) at 98 percent of the sites was scored with a severity grade of 0 (none present) and 2 percent with a score of 1 (more than 3 of the cells up to 10 percent of the area affected). Depth of necrosis (score 0 to 4) at 98 percent of the sites was scored with 0 (none present) and 2 percent were scored as 1 (upper epidermis only is affected). Vascular necrosis (score 0 to 4) at 100 percent of the sites was scored with a 0 (normal). Ulceration (absent or present) was absent at 100 percent of the sites. Hemorrhage (absent or present) for control sites was absent at 98 percent of the sites and present in 2 percent.

Clinical Observations: Edema (score 0 to 4) at 100 percent of the sites was scored as 0 (normal). Erythema description (score 0 to 3) at 100 percent of the sites was scored as 0 (none). Erythema extent (score 0-2) at 100 percent of the sites was scored as 0 (none present). Extent of necrosis (necrosis, score 0 to 4) at 100 percent of the sites was scored as 0 (none). Hemorrhage, purple discoloration was absent at 100 percent of the sites.

Biomechanical Instrumentation Measurements: Ultrasound difference from day 0 to day 2 for the 30-min Millipore water exposure sites was a mean of -0.13 ± SE 0.20. Chroma Meter difference for the same period was –0.42 ± SE 0.58. Mean Laser Doppler readings normalized to offsite controls on day 2 was 1.31 ± SE 0.07. Evaporimeter difference was a mean of –2.91 ± SE 1.46. These readings from each instrument were used for comparisons to the full-skin-thickness and partial-skin-thickness burn groups.

5.3.2.d Systemic Effects

Although systemic effects due to SM-exposure were noted in earlier experiments (Phase II) conducted under MREF Task 94-33 in hematological, clinical chemistry, and urinanalysis results presented in Phase III, no cutaneous systemic effects of SM-exposure were noted at the offsite control sites using ultrasound, Chroma Meter, Laser Doppler, or Evaporimeter instrumentation. In addition, no differences between onsite and offsite control sites were noted. Clinical chemistry and hematology data did not indicate any clinically significant effects. Therefore, the use of within animal control sites is recommended for future experiments utilizing this model and these instruments.

Histopathology: Lesions were not observed in lung and kidney sections submitted.

5.3.2.e General Lesion/burn Discussion

The full- and partial-skin-thickness wounds were not statistically significantly different from each other for the following endpoints: Chroma Meter readings, Evaporimeter readings, burn depth, wound length, hemorrhage, erythema description, erythema extent, and necrosis. Comparing the Chroma Meter results for difference of day 0 from day 2 readings between the two SM-exposed groups for the red-green balance, no differences were seen between these two groups. Lesions were deep red and covered the majority of the sites in both groups. The Chroma Meter readings for these two exposure times was statistically the same, indicating severe

erythema for both. Variation was high in depth of burn measurements since not all sites were uniformly burned to the desired depth over the exposure site. In addition, a small percentage (6%) of the 30 min SM exposure sites were not designated as full-skin-thickness. The data does indicate an increasing burn depth with the increasing exposure time. The full-skin-thickness burn exposure time should be increased greater than 30 min (~ 1 hr) for a consistent and uniform burn site within and between animals. The clinical observation endpoints, erythema description and erythema extent, likewise were graded as severe for both. Erythema was scored at or near maximum severity over the entire lesion for both exposure times at most sites. Although the Chroma Meter could not detect statistical differences between the degree of redness between these two exposure times for SM, it is a non-invasive means to measure erythema.

There were differences in response between anterior sites and the remaining sites, as reported in Phase I. Lesions at anterior sites were generally not as severe as posterior sites, nor as uniform in damage over the site. The entire area of a wound was not uniform for each endpoint at each exposure time for each animal or site. The 30-min exposure time lesions were uniform for most measurements. Histopathological endpoints such as hemorrhage, depth of necrosis related to epidermal and dermal injury, vascular injury and ulceration, were key endpoints in determining the severity of the injury. It is recommended that histopathology be included in testing results of promising therapies. Samples could be collected, but only samples from compounds or products that have been identified for continuing evaluation process.

The control and partial-skin-thickness wounds were not statistically significantly different for the following endpoints: ultrasound, burn depth, edema area, edema breadth, edema height, hemorrhage, purple discoloration, and necrosis. This was probably the result of the short exposure time and lack of uniformity of depth of burn across the exposure site and between exposure sites for the 2-min exposure time. Edema had subsided by 48 hr. In addition, the last 5 endpoints listed above are clinical observations and are more subjective even with the same evaluator. Over the course of the study, June 1999 through August 1999, several evaluators assisted, increasing the variability observed in clinical observation endpoints. Clinical observation endpoints should have the least significance during the evaluation of a product.

The control and full-skin-thickness groups were not significantly different for ulceration. Ulceration was present in 3 percent of the full-skin-thickness burn group, 25 percent of the partial-skin-thickness burn group and 0 percent for controls. Focal ulceration of the epithelium

was uncommon in the 30-min SM exposure group compared to the 2 min SM exposure group. The reason for this is unclear; it is possible that the uniform, deep, full-skin-thickness injury seen in 30-min sites decreased the microcirculation, and thus decreased the rapid influx of neutrophils and other inflammatory cells. In the early stages of injury, these cells serve to "clean up" debris, and may thus have contributed to the early focal ulcers seen principally in the 2-min sites. Over time, however, some degree of inflammatory response would be expected to occur at all sites, regardless of time of exposure, and ulceration would be expected in the 30-min sites as well. The observed differences in ulceration between the 2-min sites and the 30-min sites is interpreted as an artifact of severity of injury and time-to-sampling.

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ATTACHMENT A

Protocol 108, Amendments and Deviations

In Vivo Evaluation of Temporary Wound Dressings For Adherence, Durability and Autografting on Sulfur Mustard-Induced Lesions in Weanling Swine

Study performed by Battelle Memorial Institute
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4. Study Statistician: Ronald G. Menton, Ph.D.

5. Study Pathologist: Allen W. Singer, D.V.M.

6. Chief Veterinarian: Tracy Peace, D.V.M.

7. Sponsor: U.S. Army Medical Research and Materiel Command (USAMRMC)

8. Sponsor Monitor: LTC Richard R. Stotts, U.S. Army Medical Research Institute of Chemical Defense (USAMRICD)

9. Background: The U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) is responsible for evaluating treatment regimens to treat percutaneous bis(2-chloroethyl) sulfide (sulfur mustard; HD) exposure and for developing models to make these assessments. HD is a bifunctional alkylating agent that can produce incapacitating vesicant injury. The swine species is reported to exhibit dermal pathology similar to that observed in humans.

10. Objectives: This study is designed to develop consistent partial- to full-thickness HD-induced dermal lesions and to evaluate the use of temporary wound dressings (TWDs) for effectiveness in treating HD-induced dermal lesions in the weanling pig model. Tissue-engineered human dermal cells grown on Biobrane® II material, Dermagraft-Transitional Covering (DGTC), and other forms of TWDs may be tested. The ability of TWDs to support an autograft or promote wound healing is to be evaluated relative to a control treatment (human cadaveric skin on swine skin).

11. Experimental Design:

A. Test System

1. Animals - Specific pathogen free (SPF) hybrid porcine species from an approved SPF facility are used on the study. They are the model of choice for wound healing studies based on the structural and functional similarity to human skin. This model has limited hair cover and fixed skin, which is similar to humans. Swine also exhibit microvesication or epidermal-dermal separation following percutaneous HD exposure. The sex of the weanling pig will be recorded.

   Animals are observed throughout the course of the study. Discomfort and injury are limited to that which is unavoidable in the conduct of scientifically valuable research. If in the opinion of the Study Director or a Battelle veterinarian, an animal is in a moribund state, that animal is euthanized with an appropriate euthanasia solution. Tranquilizers, anesthetics, analgesics, and antibiotics may be used throughout the study course. Protocols of all experiments using animals are reviewed and approved by Battelle's Institutional Animal Care and Use Committee (IACUC) prior to initiation of the study.

   In Vitro methods can not replace the *in vivo* models when evaluation of physiological responses to burns, the healing process, and the multiplexity of physiologic interactions, as well as the animal's interaction with its environment serves analytical endpoints.

2. Age and Weight - The weight of the pigs at the beginning of the HD exposure period will be between 10 and 25 kg. The ages will be recorded in the study file and the age range identified in the final report.

3. Quarantine - Animals are quarantined for a minimum of 7 days. At a minimum, a routine general physical will be performed during quarantine. Animals may be scrubbed with mild soap and water, especially the entire trunk region.

4. Acclimation - If animals are quarantined at Battelle's main animal facility (505 King Ave.), then animals will be held at the MREF for at least 24 hr prior to study initiation. Animals quarantined at the MREF may be placed on study following release from quarantine.

5. Animal Identification - Animals may be ear tagged, ear marked, or tattooed in an ear to retain positive identification during animal handling and observation. Method of identification is recorded in the study file and included in the final report.

6. Dropouts and Replacements - An animal found unfit for the study during the quarantine or acclimation periods may be replaced by another animal. If an animal is replaced, any data collected on the animal is retained and the reason for replacement documented.

7. Housing - Animals may be grouped or individually housed in units meeting the requirements of Battelle SOP ARF II-012 or as directed by a Battelle veterinarian. Animal caging and/or bedding will be changed per Battelle SOP MREF VII-001.

8. Lighting - Animals are maintained under fluorescent lighting with a light/dark cycle of approximately 12 hr each per day.

9. Temperature - Air temperature in porcine rooms will be maintained in the range of approximately 50 to 80 degrees F in accordance with Battelle SOP MREF IV-001. At least 90 percent of the total twice-daily measurements will fall within this range.

10. Relative Humidity - Relative humidity in porcine rooms will be maintained in the range of approximately 30 to 70 percent in accordance with Battelle SOP MREF IV-001. At least 90 percent of the total twice-daily measurements will fall within this range.

11. Diet - Animals will be fed a commercial or producer-purchased grower or maintenance porcine ration in accordance with Battelle SOP MREF VII-003. No known contaminants that would interfere with the results of the study are known to be present in the feed. Analysis of the feed may be obtained from the commercial source or may be analyzed as deemed appropriate by the study director and/or the study veterinarian. Animals are fasted a minimum of 8 hr prior to anesthesia.

12. Water Supply - Water is supplied from the Battelle water system, and is given ad libitum during quarantine, study operation, and holding. No contaminants that would affect the results of the study are known to be present in the water. Water is not available to animals during anesthesia, but is returned once the animals have recovered.

13. Laboratory Animal Welfare Practices - Battelle's Animal Resources Facilities have been registered with the U.S. Department of Agriculture (USDA) as a Research Facility (Number 31-R-021) since 14 August 1967 and are periodically inspected in accordance with the provisions of the Federal Animal Welfare Act. In addition, animals used in research are obtained from laboratory animal suppliers duly licensed by the USDA, or reviewed and/or inspected by Battelle's Laboratory Animal Veterinarian. Battelle's statement of assurances regarding the Department of Health and Human Services (DHHS) policy on humane care of laboratory animals was accepted by the Office of Protection from Research Risks, National Institute of Health (NIH) on 27 August 1973. Animals at Battelle are cared for in accordance with the guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" (DHHS Publication No. NIH 86-23), and/or in the regulations and standards as promulgated by the Agricultural Research Service, USDA, pursuant to the Laboratory Animal Welfare Act of 24 August 1966, as amended.

14. Accreditation - On 31 January 1978, Battelle's Columbus Division received full accreditation of its animal-care program and facilities from the American Association for Accreditation of Laboratory Animal Care (AAALAC). Battelle's full accreditation status has been renewed after every inspection since the original accreditation. The MREF is a part of the facilities granted full accreditation.

B. Test Material:

1. Treatments - Appropriate documentation addressing the characteristics of the test and control articles such as the source, identification, stability, and/or storage conditions, are provided by the manufacturer or organization supplying the material. Test articles are appropriately stored per manufacturer specification and at least for the length of the study. Test articles may be evaluated for stability as directed by the sponsor.

   a. Test articles are TWDs that may include, but are not limited to Dermagraft-Transitional Covering (DGTC; Advanced Tissue Sciences), Biofill (Biofill Technologica), Graftskin (Organogenesis, Inc.), and Alloderm (Lifecell).

   b. The control article is a TWD that may include, but is not limited to, cadaveric human skin (obtained through a tissue bank) or swine skin.

2. Sulfur Mustard (HD): HD is supplied by USAMRICD. Purity, appropriate identification (batch number, lot number, state), and storage conditions are provided by USAMRICD.

   a. The purity of HD may be confirmed periodically by MREF chemists.

   b. Surety, security, and safety procedures for the use of HD are thoroughly outlined in facility safety and surety plans, in personnel requirements for qualifications to work with chemical surety materiel (CSM), and in standard operating procedures for the storage and use of CSM.

C. Study Preparations:

1. Animal Preparation Prior To Initiating The Experiment: Each study animal's weight (Section 11.E.5.) is measured to determine individual drug (preanesthetic, anesthetics, and/or medication) dosage. The animal may have the hair on its dorsum clipped and/or removed with a chemical depilatory. Next, the animal's dorsum may be gently scrubbed with a warm, mild soap solution, then rinsed with warm water and padded dry to remove debris from the trunk of the body. Prior to use of an additional pretreatment (Section 11.C.2.), the animal's dorsum may be cleaned with a bacteriostatic liquid, such as Betadine®, and then rinsed with water and then with alcohol. The animals are fasted a minimum of 8 hr prior to anesthesia.

2. Additional Pretreatment - Porcine skin can be thick enough to prevent HD from producing a partial- to full-thickness burn without prolonged exposure. To minimize the length of anesthesia and related effects, pretreatments (e.g., tape stripping or enzyme therapy) may be used to help generate a partial- to full-thickness burn.

3. Anesthesia - Animals are anesthetized during pretreatment, HD exposure, wound preparation and treatment application, and autograft application. Animals may be preanesthetized and/or masked down for intubation. Anesthesia and/or tranquilizers may be used when bandages are changed and biopsies taken.

4. Blood samples - Blood samples may be collected as needed for evaluation of wound repair and/or health status of the animal. Bleedings deemed necessary are discussed with the COR, included in the study file, and reported in the final report.

D. Test Groups: The study will be conducted in two phases with conduct of the second phase being dependant upon results of the first phase.

1. Preliminary Feasibility Study (PHASE I) - Minimally two, but not more than 16, animals are evaluated in Phase I. All wound sites are prepared as stated in Section 11.C.1. Half of the wound sites receive one of the pretreatments (Section 11.C.2.), and another pretreatment is applied to the remaining half of the wound sites. The animals are then prepared for HD exposure as described below.

a. Six dosing areas (approximately 3 cm by 3 cm) are drawn on the dorsum of anesthetized animals using the spine as the midline. HD is applied percutaneously to the dosing sites, which may be covered to aid in minimizing HD evaporation. After an HD exposure period, such as 2 hrs (to be determined), the dosed areas are decontaminated appropriately, such as with sodium hypochlorite solution followed by water rinses. After verification of decontamination, the animals are removed from the fume hood, bandaged to protect the wound area, and allowed to recover from anesthesia prior to returning to their individual cage units.

Analgesics, e.g., Banamine, may be administered to alleviate pain as directed by the Study Director and/or a Battelle veterinarian. Criteria for pain include but are not limited to the following: listless or restless behavior, anorexia, grinding of teeth, and vocalization. Antibiotics e.g., Baytril, may be used to treat infections. Any medications administered are under the direction of the Study Director and/or a Battelle veterinarian.

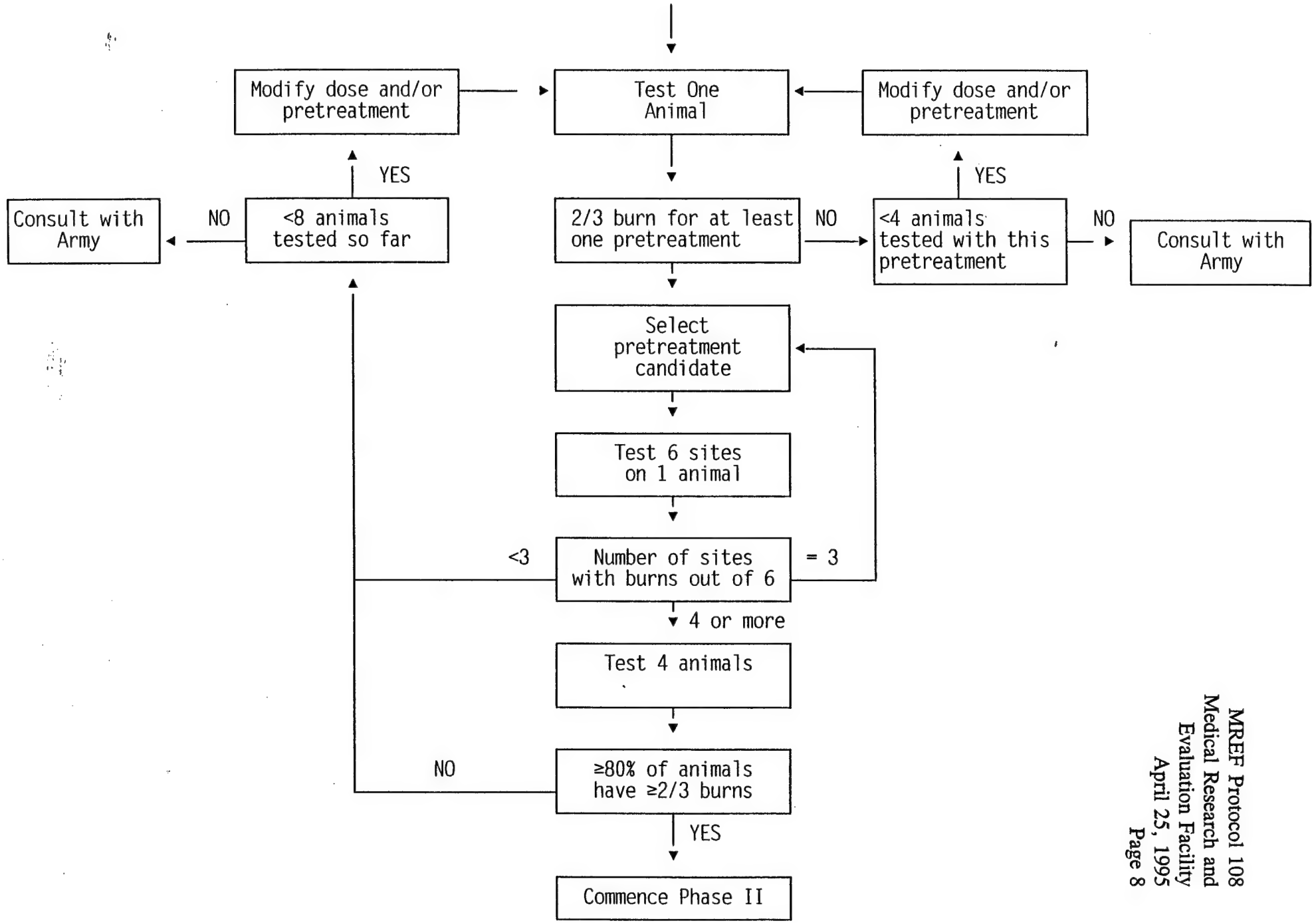
Wounds are clinically evaluated daily for approximately a week. At a minimum, one biopsy section of each lesion is taken for histological evaluation of wound severity (e.g., for 1 biopsy: 4, or 5, or 6 days post exposure; for 2 biopsies: 3 and 6 days post exposure). The biopsy(ies) will not be taken prior to the third day following HD exposure. The animals may be anesthetized or a combination of a tranquilizer and local anesthetic may be used for taking a lesion biopsy and/or during clinical evaluations.

If a pretreatment in combination with HD exposure does not produce partial- to full-thickness lesions as assessed clinically and confirmed by histopathology in 2 out of 3 sites per animal for at least 80 percent of the animals, then the evaluation of that pretreatment will be terminated.

If the combination of a specific pretreatment and HD exposure does not appear to result in a severe burn (full thickness or deep partial-thickness), then the combination may be re-evaluated and an alternate combination of pretreatment and HD exposure recommended to the COR prior to continuing. Figure 1 (on the following page) is an example of this decision tree. The resulting wounds may be debrided, and a control article implanted, followed by autograft implant even if a severe burn was not obtained. These procedures will serve to train technical staff and to define procedures in preparation for Phase II.

b. TWD Implantation - If the HD lesions are assessed clinically as partial- to full-thickness burns at the time of biopsy or by day six, then the animal is prepared for TWD application. The animal is anesthetized, bandages removed, lesions clinically evaluated, and the dosing sites cleaned with sterile saline to remove exudate. The wounds are debrided and irrigated with sterile saline to remove scabs and/or blood clots. Once the site is prepared, a TWD is applied to the wound. After TWD application, the animal is bandaged, and returned to individual housing. If human cadaveric skin is used, immunosuppressants such as Cyclosporin® (approximately 7.5 mg/kg) may be administered to minimize rejection. On the day of implant removal, adherence measures may be performed using a strain gauge to obtain a quantitative measure of implant adherence as opposed to a subjective evaluation.

After TWD implantation, bandages are changed at least two times a week and the wound sites evaluated minimally once a week. The wound sites and TWD implants are examined clinically as described in Section 11.D.1.d. Implants are left in place until the first signs of control article implant rejection or at two weeks. If clinically the wound bed appears ready for an autograft, then the animal is anesthetized and the TWD implant and granulation tissue are evaluated clinically and histopathologically as described in Sections 11.D.1.d. and 11.D.1.e, and removed. The TWD implant articles and granulation tissues may be placed in tissue fixative and appropriately identified, or handled as directed by the sponsor monitor.


Modify dose and/or pretreatment
Test One Animal
Modify dose and/or pretreatment
YES
YES
Consult with Army
NO
<8 animals tested so far
2/3 burn for at least one pretreatment
NO
<4 animals tested with this pretreatment
NO
Consult with Army
Select pretreatment candidate
Test 6 sites on 1 animal
<3
Number of sites with burns out of 6
= 3
4 or more
Test 4 animals
NO
≥80% of animals have ≥2/3 burns
YES
Commence Phase II

c. Autograft - Split-thickness pig skin autografts from the abdominal/thoracic region are maintained in an appropriate media, such as Dulbecco's Modified Eagles Medium, until application to the wound bed. The skin is prepared into a mesh (approximately 3:1), trimmed to wound size, then sutured to the skin/wound edges. Following surgery, each animal is bandaged, allowed to recover from anesthesia, and housed individually. If Cyclosporin® was administered previously, this medication can be terminated at this point. Bandages are changed at a minimum twice a week and wounds are clinically evaluated at least once a week.

At regular intervals (e.g., weekly) starting at the time of the autograft, a biopsy at the intersection of the autograft and skin bridge is taken. The sequential biopsies are taken from each wound site in a clockwise and counterclockwise fashion for dose sites on the animal's right and left sides, respectively (note: the site is considered the 12 o'clock position). The location of the first biopsy site is randomized to minimize potential experimental bias. This location of the initial biopsy site remains the same for each lesion per pig. This phase is continued for approximately four weeks following autograft application.

d. Clinical Evaluations: Clinical evaluations of wound sites are made minimally once a week after bandage removal, but may be performed more often if necessary. Routine daily animal observations will be performed as described in Battelle SOP MREF-VII-010. Clinical evaluation of wound sites include at a minimum, but are not limited to, the following events:

(1) HD Wound Sites - size of wound, exudate, erythema, edema, general impression, and severity.

(2) Test Material or Control Implants

(a) wounds - wound size, exudate, granulation, inflammation, contraction, and infection.

(b) TWD - rejection, adherence, and durability.

(3) Test Material or Control Implant wounds on the day of implant removal - criteria in Number 2 above and, in addition, erythema, edema and/or inflammation, vascularization, epithelialization, degree of wound bed preparation, and ease of removal of TWD.

(4) Autograft - wound size, adherence, re-epithelialization, necrosis, granulation, wound contraction, and infection.

(a) wounds - wound size, exudate, granulation, erythema, edema, and/or inflammation, contraction, vascularization, epithelialization, necrosis, and infection.

(b) TWD - rejection, adherence, and durability.

A clinical observation score of wound healing is determined from the clinical evaluation of the TWD and the wound for an overall wound healing completeness score. The criteria described above are used for this determination and statistical analyses.

e. Histopathology Evaluation - Biopsies are taken as described in Section 11.D.1.

(1) Biopsy evaluations for the HD exposure wounds are used to determine wound severity, such as partial- to full-thickness lesions.

(2) The remaining biopsies are evaluated using at least the following criteria: Vascularization, epithelialization, necrosis, granulation, inflammatory cell types, inflammation, and presence of test material.

In addition to (2) above, each biopsy of the autograft is evaluated for an overall wound healing score. This score is based on the histopathology criteria evaluation and made by the examining pathologist.

2. Test Article Evaluation (PHASE II): Unless otherwise specified by the sponsor monitor, two treatment sites for each type of test and control articles per animal are used. Initially, 3 types of articles may be tested (1 control and up to 2 test articles = 6 treatment sites per animal). A sequential design analyses will be performed, which may permit early termination of the study if the criteria in Section 14 are met. Up to 24 animals may be used to simultaneously evaluate test and control articles.

a. Generation of HD Lesion - The dose areas are pretreated with the best method as determined in Phase I. Animals are prepared for study as in Phase I. Analgesics and/or medications may be administered if necessary

and as determined in Phase I. Criteria for pain are explained in Section 11.D.1.a.

b. Test and Control Article Implantation - When lesions have developed sufficiently, as determined from Phase I, the animals are anesthetized, bandages removed, biopsies of the wound sites taken, and the wounds debrided. After the eschar has been excised, the implants (test article or control) will be randomly placed on the wound sites and sutured in place. Following surgery, each animal will be bandaged at least twice weekly. Clinical wound evaluations are performed at least once weekly during bandage change. Medications are administered as determined from Phase I. Clinical and histological evaluations will be performed as in Phase I.

c. Autograft: For autograft implants, the animals are anesthetized and the wound sites evaluated clinically at a time interval established for autograft implant determined from the initial feasibility study, Phase I. Clinical and histological evaluations will be performed as Phase I. The TWDs and associated granulation tissue are removed, categorized for ease of removal, placed into tissue fixative, appropriately identified, or handled as directed by the sponsor monitor. Procedures used are to be those established from Phase I results.

E. Observations and Measurements: Any person with knowledge of the test article assignments or administration of the test articles shall be excluded from collecting any observation data. This should prevent bias from knowledge of group assignment.

1. Quarantine

2. Daily Observations: The animals will be observed at the beginning and end of each work day for at least attitude, appetite, and general appearance beginning four days prior to HD exposure (Day -4). This may be included during the quarantine period.

3. Clinical Wound Evaluations: Wound evaluations will be performed at least once a week when the bandages are changed. Each TWD, autograft, and wound site is evaluated as discussed in Sections 11.D.1.d. and 11.D.1.e. If infection is suspected at the wound site, a culture may be taken for organism identification and/or antibiotic sensitivity testing.

4. Biopsies: Biopsies are performed as discussed in Sections 11.G. and 11.H.

5. Body Weight Determination: Animals will be weighed on an appropriate scale. Weights will be collected during quarantine, prior to study initiation, for medication calculations, or at the discretion of the Study Director and/or a Battelle veterinarian.

F. Photographs

Photographs may be taken of individual wound sites each time clinical evaluations are performed or if anything unusual is observed. Photographs are taken of each test site at least at the following time points:

1. Before wound creation,

2. Prior to wound excision, after wound excision, and after test article implantation,

3. Before test article removal, after test article removal, and after autograft, and

4. Before excision of wound sites.

G. Histology

1. Biopsies (approximately 4 millimeters) are taken from anesthetized, tranquilized, or euthanatized animals according to the following schedule:

   a. For Phase I, minimally one biopsy per lesion will be taken prior to test or control article implantation to evaluate lesion severity.

   b. At least four biopsies per lesion during the autograft phase are taken. Unless otherwise indicated, the biopsies are taken from the wound autograft bridge as indicated in Sections 11.D.1.d. and 11.D.2.c.

      - one biopsy prior to autograft after the test or control article and granulation tissue have been removed,

      - one biopsy at each of two separate times following autograft, and

      - at least one biopsy at study conclusion (one at the autograft and skin bridge). A second biopsy located in the center of the lesion may be taken if directed by the sponsor monitor.

2. The biopsies are placed in fixative, processed, embedded in paraffin, and stained with hematoxylin and eosin. Biopsy specimens may be subdivided and stored, or processed and stained as directed by the sponsor monitor.

3. In Phase II, the number, frequency, and time of biopsies are taken as determined from Phase I results. Biopsies to determine wound severity and at least four for autograft evaluation are taken.

H. Pathology

1. Biopsies - The examination of the wound site biopsy is limited to assessing burn severity. The remaining biopsies are examined as discussed in Section 11.D.1.e.

2. Hematology/plasma collection - A blood sample may be taken from each animal prior to wound creation, approximately seven days following HD exposure, periodically after autograft implant, and just prior to euthanasia. Blood samples are placed in vacutainer tubes containing an appropriate anticoagulant. Differential counts may be performed on a portion of the specimen collected and the remainder is centrifuged and the resulting plasma stored at less than -10 degrees C for up to one year following study termination or as directed by the sponsor monitor. The plasma may be examined for antibodies formed against the test material if an inflammatory or rejection response was noted.

12. Dead or Moribund Animals: Animals that are judged to be moribund may be euthanatized and appropriate skin samples collected. A gross necropsy may be performed as directed by the sponsor monitor. If an animal dies on study, an attempt may be made to determine if the death is test article related. The sponsor is notified immediately and further instructions sought. At a minimum, the test article/test sites will be removed along with the animal's identification, and placed in a freezer-storage bag for placement into an approximately 70 degrees C freezer. Evaluation of the test article/test sites will be performed as directed by the sponsor monitor. Animals may be replaced during the study at the discretion of the Study Director after consultation with the sponsor monitor.

13. Study Termination: At the termination of the study, animals will be euthanatized in accordance with guidelines set forth by the American Veterinary Medical Association Panel on Euthanasia. Animal carcasses will be disposed of by incineration. After euthanasia, final biopsy samples for histopathological examination may be taken and the remaining test or control sites may be excised. One-half may be snap frozen or handled as directed by the sponsor monitor, and the other half placed in tissue fixative. Samples are appropriately labeled and will be sent to the sponsor or the sponsor's designee. Specimens are examined for inflammatory response, granulation tissue, vascularization, epithelialization, and presence of test article.

14. Statistical Analyses

Statistical analyses will focus on four primary parameters: overall wound severity score (WSS), clinical wound healing score (CWHS), histological wound healing score (HWHS), and wound size. Statistical analysis may also be performed on selected individual components of CWHS and HWHS, such as re-epithelialization or infection.

To minimize animal usage in Phase II, experiments are performed using a two-stage, group sequential hypothesis test[1]. The first stage consists of the experimental results for 12 animals. An interim analysis is performed using the data from these 12 animals. If the efficacy of a TWD is significantly less than that of the control treatment, then the evaluation is considered complete and no more animals are treated with this TWD. Otherwise, up to 12 additional animals will be tested, and the efficacy of the TWD relative to that of the control treatment is reassessed. The significance levels of the interim and final analyses are carefully controlled to maintain an overall Type 1 error rate of 0.05. This may be accomplished by selecting significance levels of 0.001 and 0.05 for the interim and final analyses, respectively. Because HWHS data are not immediately available, the hypothesis test is conducted using the CWHS data only.

Overall wound severity score: For Phase I experiments, WSSs are statistically analyzed to determine the pretreatment (none, tape stripping, or enzyme therapy) that most consistently generates a partial- to full-thickness burn. Mean WSS of pretreated sites is compared to those that were not pretreated using an analysis of variance (ANOVA) model. The assumption of approximate normality for the distribution of WSSs is assessed visually. If this assumption is grossly violated, then either a transformation will be applied to the data prior to fitting the ANOVA models, or the analysis will be conducted using nonparametric or categorical methods.

For Phase II experiments, wound size and severity scores are statistically compared among the temporary wound dressings. The purpose of the analysis is to assess whether or not baseline wounds are similar among the TWDs. If statistical differences ($p=0.05$) are found between baseline wounds for the TWDs, then baseline wound size or severity score may be used as a covariate in efficacy comparisons between TWDs.

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[1] Geller, N.L. and Pocock, S.J. (1988). "Design and Analysis of Clinical Trials with Group Sequential Stopping Rules" in Biopharmaceutical Statisitics for Drug Development, Edited by Peace, K.E., New York, Marcel Dekker.

Clinical wound healing score: For Phase I experiments, test or control articles may be implanted for training purposes. Descriptive statistics and plots are prepared to assess the variability between animals and over time in CWHSs.

For Phase II experiments, CWHSs are statistically compared among the TWDs. The overall Type I error rate for the stagewise hypothesis test is controlled at the 0.05 level. Mean CWHS of each TWD is compared to that of the control treatment to determine if the TWD is as effective in treating HD-induced dermal lesions. If multiple sites are given the same treatment (TWD or control) in the same animal, then data may be averaged to provide a single score for each animal and treatment. Based on the evaluation of Phase I data, evaluations in Phase II may be performed on transformed data. Statistical comparisons are performed using either an ANOVA test or a one-sided paired Student t-test. Analyses may be performed using analogous nonparametric methods. If significant differences are found between baseline wounds for the TWD and control treatment, then the baseline wound size or severity score may be used as a covariate in the ANOVA tests.

Histological wound healing score: Statistical analysis of HWHS data will be the same as that conducted on CWHS data. Because HWHS data are not immediately available, a single hypothesis test, rather than a two-stage hypothesis test, is conducted upon completion of experimental tests for each TWD.

Wound size: For Phase I experiments, descriptive statistics and plots are prepared to assess the variability in wound size within animals, between animals, and over time.

For Phase II experiments, mean wound size of each TWD is statistically compared to that of the control treatment using an ANOVA model. Two-sided tests are performed at the 0.05 significance level. A repeated measures ANOVA model may be used. The assumption of approximate normality for the distribution of wound size is assessed visually. If this assumption is grossly violated, then either a transformation may be applied to the data prior to fitting the ANOVA models, or the analysis may be conducted using nonparametric methods.

15. Adverse Reactions: Any adverse reactions observed during the study are recorded. All deaths (test article related or not) are reported immediately to the sponsor monitor.

16. Record Maintenance: The following records are to be maintained:

A. CSM accountability log and inventory,

B Test article inventory and related information,

C. Reagent preparation,

D. Animal receipt and quarantine records,

E. Animal data from tests performed,

F. Decontamination and disposal records, and

G. Any other records needed to reconstruct the study and demonstrate adherence to this protocol.

17. Reports:

A. Draft Final Report: A draft final report is prepared within 30 working days after receipt of data analyses and evaluations. The draft report includes:

1. Experimental design,

2. Animal husbandry,

3. Test article description(s),

4. Clinical observations of wounds,

5. Histopathological data summary,

6. Statistical analysis of data, and

7. Discussion and conclusion.

B. Final Report - Following receipt of comments on the draft final report from USAMRMC, a final report will be prepared within 30 working days.

18. Approval Signatures:

Frances M. Reid — 4-25-95

Frances M. Reid, D.V.M.
Study Director — Date

Robyn C Kiser — 4-25-95

Robyn C. Kiser, B.S.
Task Leader — Date

John B. Johnson — 25 April 1995

J. Bruce Johnson, D.V.M.,
Co-Principal Investigator and Manager
Medical Research and Evaluation Facility — Date

Ronald G. Menton — 4-25-95

Ronald G. Menton, Ph.D.
Study Statistician — Date

Allen W. Singer — 4-28-95

Allen W. Singer, D.V.M.
Study Pathologist — Date

for T.A. Peace

042895

Tracy Peace, D.V.M.
Chief Veterinarian

Date

10 JUL 95

LTC Richard R. Stotts, VC
USAMRICD COR

Date

In Vivo Evaluation of Temporary Wound Dressings For Adherence, Durability and Autografting on Sulfur Mustard-Induced Lesions in Weanling Swine

MREF Protocol 108. Amendment No. 1

Change Number No. 1

Change: Delete the following paragraph under section 10. Objectives: on page 1 of MREF Protocol 108.

> This study is designed to develop consistent partial- to full thickness HD-induced dermal lesions and to evaluate the use of temporary wound dressings (TWDs) for effectiveness in treating HD-induced dermal lesions in the weanling pig model. Tissue-engineered human dermal cells grown on Biobrane® II material, Dermatograft-Transitional Covering (DGTC), and other forms of TWDs may be tested. The ability of TWDs to support an autograft or promote wound healing is to be evaluated relative to a control treatment (human cadaveric skin or swine skin).

Insert the following paragraph in place of.the one above under section 10. Objectives: on page 1 of MREF Protocol 108.

> This study is designed to develop consistent partial- to full thickness HD-induced dermal lesions and to evaluate the use of a temporary wound dressing (TWD) for effectiveness in treating HD-induced dermal lesions in the weanling pig model. Tissue-engineered human dermal cells grown on Biobrane® II material, Dermatograft-Transitional Covering (DGTC), will be tested. The ability of the TWD to support an autograft or promote wound healing is to be evaluated relative to a control treatment (human cadaveric skin or swine skin).

Reasons for Changes:

> DGTC is the only product that is being tested.

Impact on the Study:

> There are no adverse affects on the study nor is the integrity of the study compromised.

Change No. 2

Change: Delete the following paragraph under the Test Material: Section 11. B. 1. a. on page 4 of MREF Protocol 108.

> Test articles are TWDs that may include, but are not limited to Dermatograft-Transitional Covering (DGTC; Advanced Tissue Sciences), Biofill, (biofill Technologica), Graftskin (Organogenesis, Inc.), and Alloderm (Lifecell).

Insert the following paragraph under the Test Material: Section 11. B. 1. a. on page 4 of MREF Protocol 108.

> The test article is Dermatograft-Transitional Covering (DGTC) provided and manufactured by Advanced Tissue Sciences.

Reasons for Change:

DGTC is the only product that is being tested.

Impact on the Study:

There are no adverse affects on the study nor is the integrity of the study compromised.

Change No. 3

Change: Delete the following first two sentences of section 11. D. 2. Test Article Evaluation (Phase II) on page 10 of MREF Protocol 108.

> Unless otherwise specified by the sponsor monitor, two treatment sites for each type of test and control articles per animal are used. Initially, 3 types of articles may be tested (1 control and up to 2 test articles = treatment sites per animal).

Insert the following sentences as the first two sentences of section 11. D. 2. Test Article Evaluation (Phase II).

> Unless otherwise specified by the sponsor monitor, two treatment sites for control articles and four treatment sites for DGTC per animal are used (a total of 6 treatment sites per animal).

Reasons for Change:

DGTC is the only product that is being tested.

Impact on the Study:

There are no adverse affects on the study nor is the integrity of the study compromised.

Change No. 4

Change: Delete the following paragraph in Statistical Analyses section 14. paragraph 2 under Overall wound severity score: on page 14 of MREF Protocol 108.

> For Phase II experiments, wound size and severity scores are statistically compared among the temporary wound dressings. The purpose of the analysis is to assess whether or not baseline wounds are similar among the TWDs. If statistical differences ($p=0.05$) are found between baseline wounds for the TWDs, then baseline wound size or severity score may be used as a covariate in efficacy comparisons between TWDs.

Insert the following paragraph in place of the one above under Statistical Analyses section 14. paragraph 2 under Overall wound severity score: on page 14 of MREF Protocol 108.

> For Phase II experiments, wound size and severity scores are statistically compared between the TWD and control article. The purpose of the analysis is to assess whether or not baseline wounds are similar between the test and control article. If a statistical difference ($p=0.05$) is found, then baseline wound size or severity score may be used as a covariate in efficacy comparisons.

Reason for Change:

DGTC is the only product that is being tested. The statistical analyses will occur between the control article and DGTC.

Impact of Change:

There are no adverse affects on the study nor is the integrity of the study compromised.

Change No. 5

Change: Delete the following paragraph in the Statistical Analyses section 14. paragraph 2 under Clinical wound healing score: on page 15 of MREF Protocol 108.

> For Phase II experiments, CWHSs are statistically compared among the TWDs. The overall Type I error rate for the stagewise hypothesis test is controlled at the 0.05 level. Mean CWHS of each TWD is compared to that of the control treatment to determine if the TWD is as effective in treating HD-induced dermal lesions. If multiple sites are given the same treatment (TWD or control) in the same animal, then data may be averaged to provide a single score for each animal and treatment. Based on the evaluation of Phase I data, evaluations in Phase II may be performed on transformed data. Statistical comparisons are performed using either an ANOVA test or a one-sided paired Student t-test. Analyses may be performed using analogous nonparametric methods. If significant differences are found between baseline wounds for the TWD and control treatment, then baseline wound size or severity score may be used as a covariate in the ANOVA tests.

Insert the following paragraph in place of the one above under the Statistical Analyses section 14. paragraph 2 under Clinical wound healing score: on page 15 of MREF Protocol 108.

> For Phase II experiments, CWHSs are statistically compared between the test and control article. The overall Type I error rate for the stagewise hypothesis test is controlled at the 0.05 level. Mean CWHS of the test article is compared to that of the control to determine if the test article is as effective in treating HD-induced dermal lesions. If multiple sites are given the same treatment (TWD or control) in the same animal, then data may be averaged to provide a single score for each animal and treatment. Based on the evaluation of Phase I data, evaluations in Phase II may be performed on transformed data. Statistical comparisons are performed using either an ANOVA test or a one-sided paired Student t-test. Analyses may be performed using analogous nonparametric methods. If significant differences are found between baseline wounds for the test article and control articles, then baseline wound size or severity score may be used as a covariate in the ANOVA tests.

Reason for Change:

DGTC is the only product that is being tested. The statistical analyses will occur between the control article and DGTC.

Impact of Change:

There are no adverse affects on the study nor is the integrity of the study compromised.

Change No. 6

Change: Delete the following the last three words from the paragraph under Histological wound healing score: under the Statistical Analyses section 14. on page 15 of MREF Protocol 108.

for each TWD

Reason for Change:

DGTC is the only product that is being tested. The statistical analyses will occur between the control article and DGTC. Thus, there is only one TWD.

Impact of Change:

There are no adverse affects on the study nor is the integrity of the study compromised.

Change No. 7

Change: Delete the words "each TWD" in the first sentence of the second paragraph under Wound size: under the Statistical Analyses section 14. on page 15 of MREF Protocol 108 and replace with "the test article".

Reason for Change:

DGTC is the only product that is being tested. The statistical analyses will occur between the control article and DGTC. Thus, only one test article will be evaluated.

Impact of Change:

There are no adverse affects on the study nor is the integrity of the study compromised.

Frances M. Reid
Frances M. Reid
Study Diirector

1-19-96
Date

LTC Richard R. Stotts, COR
USAMRICD

23 JAN 96
Date

In Vivo Evaluation of Temporary Wound Dressings For Adherence, Durability and Autografting on Sulfur Mustard-Induced Lesions in Weanling Swine

MREF Protocol 108. Amendment No. 2

Addition: Add the following section on page 10 under Section 11.D.1.f.: Scoring regimen for clinical observations and histological evaluations of wound sites.

Clinical Observations and Histological Evaluations of the Wound Site

This section defines and presents a minimal scoring regimen to use on each lesion for this study. This scoring regimen may need to be adjusted based on observations made during Phase I. Any changes or adjustments will be following discussions and approval by the COR and/or sponsor designee.

I. Definitions

Clinical Observations and Histological Evaluations of the Wound Site

This section defines and presents a minimal scoring regimen to use on each lesion for this study. This scoring regimen may need to be adjusted based on observations made during Phase I. Any changes or adjustments will be following discussions and approval by the COR and/or sponsor designee.

I. Definitions

Adherence -	the act or quality of sticking to something.
Contraction/Closure -	a drawing together, a shortening or shrinkage.
Durability -	highly resistant to wear and tear.
Edema -	presence of large amounts of fluid in intercellular spaces of the body. Edema encompasses swelling.
Epithelialization -	healing by the growth of epithelium over a denuded surface.

Erythema - a name applied to the redness of the skin produced by congestion of the capillaries, which may result from a variety of causes.

Eschar - a slough produced by a thermal burn, corrosive application, or by gangrene.

Exudate - material, such as fluid, cells or cellular debris, which has escaped from blood vessels and is deposited in or on tissues. Exudate are characterized by high protein content, cells, or solid materials derived from cells.

Granulation - the formation in wounds of small, rounded masses of tissue during healing.

Inflammation - a localized protective response elicited by injury or destruction of tissues, which serves to destroy, dilute, or wall off both injurious agent and the injured tissue. Inflammation is characterized by pain, heat, redness, and edema.

Necrosis - the sum of morphological changes indicative of cell death and caused by the progressive degradetive action of enzymes.

Rejection - the process of walling off and/or failure to incorporate foreign material. Graft rejection is an immune response against a grafted tissue that results in the failure of the graft to survive. Graft rejection is characterized histologically as an extensive infiltration by mononuclear cells, primarily small lymphocytes, accompanied by edema and interstitial hemorrhage. Observational rejection will be characterized as nonvascularized material with lack of adherence and accompanied by necrosis.

Sloughing - the formation or separation of necrotic tissue in the process of separating from viable portions of the body. To shed or cast off.

Swelling/Edema -	a transient abnormal enlargement or increase in volume of a body part or area not caused by proliferation of cells.
Vascularization -	the formation of new blood vessels.
NA -	Score NA when observations an not be made and give justification.

II. Observation and Histological Evaluation and Scoring of Each Lesion by Event

The scoring criteria and criteria descriptions may need to be changed, based on observations during Phase I.

A. Wound Site

A-1. Clinical Observation - Initially daily observations are made until adequate observation intervals are identified.

1. Size of Wound - Metric measurement with ruler

2. Exudate

0	None	
1	Minimal -	Less than 1/3 of the wound area is covered.
2	Moderate -	Between 1/3 and 2/3 of the wound area is covered.
3	Maximum - Greater than 2/3 of the wound area is covered.	

The following observations of the wound area should occur after gentle cleaning.

3. Erythema

0	None	
1	Slight -	Light pink to pink, area not well defined.
2	Slight to Moderate -	Pink to light red, well defined area

3	Moderate -	Red, well defined lesion.
4	Moderate to Severe -	Red to deep red (beet red), well defined lesion to spreading = possibly larger than original site.
5	Severe -	Deep red to purple, evidence of necrosis and/or eschar in addition to criteria for 4 above.

4. Edema

0	None	
1	Slight -	Barely perceptible or questionable.
2	Slight to Moderate -	Slightly raised area with well defined edges.
3	Moderate -	Area raised approximately 1 millimeter, well defined.
4	Moderate to Severe -	Area raised greater than 1 millimeter, well defined possibly spreading larger than original site.
5	Severe -	Area raised greater than 1 millimeter and extending beyond area of exposure.

5. General Impression - Overall observer's impressions.

0	None	
1	Slight -	Lesion maybe slightly erythematous or slightly edematous with area not well defined.
2	Slight to Moderate -	Lesion may be erythematous and/or edematous in a well defined area.
3	Moderate -	Lesion may be severely erythematous and/or severely edematous in a well defined or beginning to spread area.
4	Moderate to severe -	Lesion may be severely edematous, severely erythematous, and/or spreading with some evidence of necrosis (exudate).
5	Severe -	Lesion with severe necrosis and/or eschar and may have spread.

A-2. Clinical Observational Wound Severity Score - Function of scores from criteria 2 through 5.

A-3. Histology Evaluation - The wound biopsy is evaluated for full-thickness or deep-partial thickness burn.

B. Test Material or Control Implants and Wounds

B-1. Clinical Wound Healing Observation of Wound Area - Minimally once a week during bandage change.

1. Wound Size - Metric measurement with ruler

2. Exudate - See section II.A.A-1.2.

The following observations of the wound area should occur after gentle cleaning.

3. Granulation

0	None
1	Minimal
2	Moderate
3	Maximum

4. Inflammation

0	None -	No inflammation observed.
1	Slight -	Barely perceptible, light pink to pink, not well defined.
2	Slight to Moderate -	Slightly raised area with well defined edges, pink to light red.
3	Moderate -	Area light red to red and raised approximately 1 millimeter, well defined.
4	Moderate to Severe -	Area red to deep red and raised greater than 1 millimeter, well defined possibly spreading larger than original site.

5	Severe -	Area deep red to purple with evidence of necrosis or eschar and raised greater than 1 millimeter and extending beyond area of exposure.

5. Contraction

0	Maximum
1	Moderate
2	Minimal
3	None

6. Infection

0	Absent
1	Present

B-2. Clinical Observations of Test or Control Articles - Minimally once a week during bandage change.

1. Rejection

0	None -	No rejection
1	Very slight -	Isolated, small areas, less than a quarter of the lesion, indicates material rejection.
2	Slight -	Approximately one quarter to half of the lesion indicates material rejection.
3	Moderate -	Over half of the lesion indicates material rejection.
4	Extensive -	Rejection of material.

2. Adherence

0	No slough -	Adhered, entire TWD is adhered to the lesion.
1	Very slight -	Isolated, small areas less than a quarter of the lesion is sloughing.

2	Slight -	Approximately one quarter to half of the lesion is sloughing.
3	Moderate -	Over half of the lesion is sloughing.
4	Extensive -	Entire lesion sloughed.

3. Durability

0	Extensive durability -	Greater than 2/3 of wound area durable.
1	Moderate durability -	Between 1/3 and 2/3 of wound area durable.
2	Minimal durability -	Less than 1/3 of wound area durable.
3	None -	Total breakdown of TWD

B-3. Clinical Observation Wound Healing Score - Function of scores from B-1., except for Nos. 1., and B-2.

B-4. Histology Evaluation - None.

C. Test or Control Article Removal Day - Removed maximally 2 weeks after implant or earlier if control article is sloughing.

C-1. Clinical Observations of Test or Control Wound Site

1. Wound Size - Metric measurement with ruler

2. Exudate - See section II.A.A-1.2.

The following observations of the wound area should occur after gentle cleaning.

3. Erythema - See section II.A.A-1.3.

Note: This may be deleted if found not relevant or well represented by the criteria section inflammation.

4. Edema - See section II.A.A-1.4.

Note: This may be deleted if found not relevant or well represented by the criteria section inflammation.

5. Granulation - See section II.B.B-1.3.

6. Inflammation - See section II.B.B-1.4.

7. Vascularization

0	Extensive
1	Moderate to Extensive
2	Moderate
3	Slight to Moderate
4	Very Slight
5	None

8. Epithelialization - Rough estimation of percent of closure.

0	Extensive -	100 percent closed.
1	Moderate to Extensive -	75 to 100 percent closed.
2	Moderate -	50 to 75 percent closed.
3	Slight to Moderate -	25 to 50 percent closed.
4	Very Slight -	0 to 25 percent closed.
5	None -	0 percent closed.

9. Contraction - See section II.B.B-1.5.

10. Infection - See section II.B.B-1.6.

11. Degree of Wound Bed Preparation

0	None
1	Minimal
2	Moderate
3	Extensive

C-2. Clinical Observations of Test or Control Article

1. Rejection - See section II.B.B-2.1.

2. Adherence - See section II.B.B-2.2.

3. Durability - See section II.B.B-2.3.

4. Ease of removal of Test or Control Article - Maybe quantitative and scored.

0	Easily Removed - No adherence or sloughed.	
1	Slight adherence -	Gentle pull removes material.
2	Moderate -	Firm pull and some cutting may be required.
3	Difficult -	Material must be dissected out.

C-3. Clinical Observation Wound Healing Score - Function of C-1, except Nos. 1, and C-2 scores.

C-4. Histology Evaluation - Of removed test article or control implant and site.

1. Inflammation - Include cell types

0	None -	No inflammation observed.
1	Slight	
2	Slight to Moderate	
3	Moderate	
4	Moderate to Severe	
5	Severe	

2. Vascularization

0	Extensive
1	Moderate to Extensive
2	Moderate
3	Slight to Moderate
4	Very Slight
5	None

3. Epithelialization

0	Extensive
1	Moderate to Extensive
2	Moderate
3	Slight to Moderate
4	Very Slight
5	None

4. Necrosis

0	None
1	Slight
2	Moderate
3	Severe

5. Granulation

0	None
1	Minimal
2	Moderate
3	Maximal

6. Presence of Test or Control Material

0	Absent
1	Present

7. Completeness of Wound Healing Determined by Pathologist

0	Maximum
1	Moderate
2	Minimal
3	None

D. Autograft

D-1. Clinical Observations of Autograft - Minimally once a week during bandage change.

1. Wound Size - Metric measurement with ruler

2. Exudate - See section II.A.A-1.2.

The following observations of the wound area should occur after gentle cleaning.

3. Rejection - See section II.B.B-2.1.

4. Adherence - See section II.B.B-2.2.

5. Durability - See section II.B.B-2.3.

6. Erythema - See section II.A.A-1.3.

   Note: This may be deleted if found not relevant or well represented by the criteria section inflammation.

7. Edema - See section II.A.A-1.4.

   Note: This may be deleted if found not relevant or well represented by the criteria section inflammation.

8. Granulation - See section II.B.B-1.3.

9. Inflammation - See section II.B.B-1.4.

10. Vascularization - See section II.C.C-1.7.

11. Epithelialization - See section II.C.C-1.8.

12. Necrosis

0	None	
1	Minimal -	Less than 1/3 of the wound area is covered.
2	Moderate -	Between 1/3 and 2/3 of the wound area is covered.
3	Maximum -	Greater than 2/3 of the wound area is covered.

13. Contraction - See section II.B.B-1.5.

14. Infection - See section II.B.B-1.6.

D-2. Clinical Observation Wound Healing Score - Function of D-1 scores except for .

D-3. Histology Evaluation - Weekly biopsies.

1. Inflammation - See section II.C.C-4.1.

2. Vascularization - See section II.C.C-4.2.

3. Epithelialization - See section II.C.C-4.3.

4. Necrosis - See section II.C.C-4.4.

5. Granulation - See section II.C.C-4.5.

6. Presence of Test or Control Material - See section II.C.C-4.6.

7. Completeness of Wound Healing Determined by Pathologist - See section II.C.C-4.7.

Reason for Addition: To elaborate on a scoring regimen used for lesion evaluation on this study.

Impact on Study: To aid in scoring lesions in a consistent manner.

Frances M. Reid
Frances M. Reid
Study Diirector

4-16-96
Date

Richard R. Stotts
LTC Richard R. Stotts, COR
USAMRICD

16 APR 96
Date

In Vivo Evaluation of Temporary Wound Dressings For Adherence, Durability and Autografting on Sulfur Mustard-Induced Lesions in Weanling Swine

MREF Protocol 108. Amendment No. 3

Change 1. In the title and throughout the protocol, wherever HD or sulfur mustard is mentioned, change to read:

"HD or other vesicant/irritant"

Reason for Change: To develop a consistent partial- to full-thickness chemical burn, it may be necessary to use another vesicant or some other irritant. These will be obtained, stored, handled, and disposed of according to appropriate safety practices and guidelines.

Impact on Study: None.

Change 2. Throughout the protocol whenever dosing on the pig's dorsum is mentioned, change this to read that dosing may occur on either the dorsal or ventral surface of the animal.

Reason for Change: To develop a consistent partial- to full-thickness chemical burn, it may be necessary to dose either the dorsal or ventral area of the trunk of the animal.

Impact on Study: None.

Change 3. Page 2. Section 11.A.4. Change the first sentence to read:

"Acclimation - If animals are quarantined at Battelle's main animal facility (505 King Ave.), then animals will be held at the MREF for at least 48 hr prior to study initiation."

Reason for Change: To allow a longer acclimation period for the animals if necessary.

Impact on Study: None.

Change 4. Page 6. Section 11.D.1.a. Insert before and change the last sentence of the first paragraph to read:

"During this feasibility study, an efficient method of decontamination will be developed, along with satisfactory proof of decontamination procedures. Wound Sites may or may not be bandaged."

Reason for Change: The actual procedures employed for the decontamination of the dosed sites will need to be developed as will proof of decontamination procedures. Also, at the discretion of the study director or staff veterinarian, it may not be necessary to bandage the wound area on some animals. Healing may occur satisfactorily without bandaging.

Impact on Study: None.

Change 5. Page 6. Section 11.D.1.a. In paragraph three, delete the parenthesis and inclusion of the second sentence through the third sentence, so that the paragraph reads:

"Wounds are clinically evaluated daily for approximately a week. At a minimum, one biopsy section of each lesion is taken for histological evaluation of wound severity. The animals may be anesthetized, or a combination of a tranquilizer and local anesthetic may be used for taking a lesion biopsy and/or during clinical evaluations."

Reason for Change: In order to follow wound development, biopsies may be needed within hours of the HD exposures.

Impact on Study: None.

Change 6. Page 7. Section 11.D.1.b. Change first sentence to read:

"If the lesions are assessed clinically as partial- to full-thickness burns at the time of biopsy, then the animal is prepared for TWD application."

Reason for Change: Biopsies may be taken after day 6 as determined by the study director or staff veterinarian.

Impact on Study: None.

Change 7. Page 9. Section 11.D.1.d.(1). Add:

"Wound Sites - size of wound, exudate, erythema, edema, necrosis, eschar, general impression, and severity."

Reason for Change: To add necrosis and eschar parameters to describe wound evaluation.

Impact on Study: To aid in a more precise wound description.

Change 8. Page 13. Section 11.H.2. Add as a last sentence:

"It is not anticipated that blood samples will be taken at this time. If, however, sampling is necessary, the study director will notify the attending veterinarian of the blood volume required and ask for IACUC approval."

Reason for Change: To clarify that if blood samples are drawn, IACUC and attending veterinarian approval will be required.

Impact on Study: None.

Change 9. Amendment No. 2. Page 28. Section II.A.A-1. Insert 5 and 6 as follows:

"5 Necrosis

0 None
1 Slight/minimum
2 Slight to Moderate
3 Moderate
4 Severe/maximum

6 Eschar

0 Absent
1 Present"

Change numbering of "General Impression" to 7.

Reason for Change: To add two parameters to describe wound evaluation.

Impact on Study: To aid in a more precise wound description.

Change 10. Amendment No. 2. Page 29 and 30. Section II.B.B-1.4. Change 3, 4, and 5 to:

"3 Moderate - Area light red to red and may be raised, well defined.

4 Moderate to Severe - Area red to deep red and may be raised, well defined and spreading beyond the original site.

5 Severe - Area deep red to purple with necrosis or eschar and may be raised and extended beyond area of exposure."

Reason for Change: It is difficult to evaluate lesions as being raised approximately 1 mm.

Impact on Study: None.

Change 11. Amendment No. 2. Page 32. Section II.C.C-1.7. Change rating scale to read:

"0 None
1 Very Slight
2 Slight to Moderate
3 Moderate
4 Moderate to Extensive
5 Extensive"

Reason for Change: To clarify the vascularization rating scale.

Impact on Study: None.

Change 12. Amendment No. 2. Page 33. Section II.C.C-4.2. Change rating scale to read:

"0 None
1 Very Slight
2 Slight to Moderate
3 Moderate
4 Moderate to Extensive
5 Extensive"

Reason for Change: To clarify the vascularization rating scale.

Impact on Study: None.

Change 13. Amendment No. 2. Page 34. Section II.C.C-4.4. Change rating scale to read:

"0 None
1 Slight/minimum
2 Slight to Moderate
3 Moderate
4 Severe/maximum"

Reason for Change: To clarify the necrosis rating scale.

Impact on Study: None.

Frances M. Reid
Frances M. Reid
Study Diirector

6-7-96
Date

LTC Richard R. Stotts, COR
USAMRICD

10 Jun 96
Date

In Vivo Evaluation of Temporary Wound Dressings For Adherence, Durability and Autografting on Sulfur Mustard-Induced Lesions in Weanling Swine

MREF Protocol 108. Amendment No. 4

Change Number 1:

Change: Delete the third paragraph under section 11. Experimental Design D. 1. a. on page 6 of MREF Protocol 108.

> Wounds are clinically evaluated daily for approximately a week. At a minimum, one biopsy section of each lesion is taken for histological evaluation of wound severity (e.g., for 1 biopsy: 4, or 5, or 6 days post exposure; for 2 biopsies: 3 and 6 days post exposure). The biopsy(ies) will not be taken prior to the third day following HD exposure. The animals may be anesthetized or a combination of a tranquilizer and local anesthetic may be used for taking a lesion biopsy and/or during clinical evaluations.

Insert the following paragraph in the above paragraph's place in section 11. Experimental Design D. 1. a.

> Wounds may be clinically evaluated daily for approximately a week. At a minimum, one biopsy section of each lesion is taken for histological evaluation of wound severity (e.g., for 1 biopsy: Either 1, 2, 3, 4, 5, or 6 days post exposure; for 2 biopsies: 3 and 6 days post exposure). The biopsy(ies) may be taken as early as 24 hrs following HD exposure. The animals may be anesthetized or a combination of a tranquilizer and local anesthetic may be used for taking a lesion biopsy and/or during clinical evaluations.

Reasons for Changes:

During development of the wound, biopsies taken earlier than day three were necessary to evaluate wound development. Consultation with the Contract Officer Representative (COR) determined that biopsies may need to be taken prior to day 3.

Impact on the Study:

There are no adverse affects on the study nor is the integrity of the study compromised.

Change No. 2

Change: Delete the following sentence in paragraph 2 of TWD Implantation, under the Test Groups section 11. D. 1. b. on page 7 of MREF Protocol 108.

After TWD implantation, bandages are changed at least two times a week and the wound sites evaluated minimally once a week.

Insert the following sentences in place of the above sentence in paragraph 2 of TWD Implantation, under the Test Groups section 11. D. 1. b. on page 7 of MREF Protocol 108.

After TWD implantation, bandages may be applied minimally once. If additional bandaging is required, bandages are changed at least two times a week. The wound sites are evaluated minimally once a week.

Reasons for Change:

Preliminary implantaion results, during training, indicated that the wound tended to heal with less infection and healing irritation to the animal when the bandage was removed.

Impact on the Study:

There are no adverse affects on the study nor is the integrity of the study compromised. This may improve the healing ability of the wound site by providing direct air (oxygen) contact.

Change No. 3

Change: Delete the following sentence in the middle of the paragraph under b. Test and Control Article Implantation in section 11. D. 2. Test Article Evaluation (Phase II):

Following surgery, each animal will be bandaged at least twice weekly.

Insert the following sentences in place of the above sentence:

Each animal will be bandaged immediately after surgery. Additional bandaging and bandage changes may be necessary.

Reasons for Change:

Preliminary wound dressing applications on dosed and TWD/autografted animals indicated that the wound sites were less traumatized and fewer infections if left to the open air.

Impact on the Study:

There are no adverse affects on the study nor is the integrity of the study compromised.

Change No. 4

Change: Delete the words "are" and "at least" in the second sentence of section 11.F. Photographs. Replace are with "may be" so that sentence reads as follows:

Photographs may be taken of each test site at the following time points:

Reason for Change:

Some of these pictures may be unnecessary. Until the entire procedure is performed, it is difficult to know when to take the photographs. Portions of Phase I will help in determining what pictures and when to take pictures.

Impact of Change:

There are no adverse affects on the study nor is the integrity of the study compromised.

Frances M. Reid

Frances M. Reid
Study Diirector

9-16-96
Date

Richard R. Stotts

LTC Richard R. Stotts, COR
USAMRICD

16 SEP 96
Date

In Vivo Evaluation of Temporary Wound Dressings For Adherence, Durability and Autografting on Sulfur Mustard-Induced Lesions in Weanling Swine

MREF Protocol 108. and the 4 Amendments Amendment No. 5

Change Number 1:

Change: Throughout the protocol and four amendments where ever TWD or TWDs is written; replace with "test article".

Reasons for Change: The sponsor modified the study design to resolve study issues, simplify the study, reduce study variables encountered during method development, and align the study to meet the sponsor's needs.

Impact on the Study: There are no adverse affects on the study nor is the integrity of the study compromised.

Change Number 2:

Change: Throughout the protocol and four amendments wherever bandaging or the frequency of bandaging is mentioned, insert the statement that "Bandaging techniques and frequency of bandaging will be determined in Phase I. Examples of frequency are as follows:".

Reasons for Change: During Phase I, bandaging of wounds after autograft and TWD application indicated an alternate method was needed since, adherence of the autograft and TWD was minimal or not at all and inconsistent. The sponsor demonstrated alternate methods of applying the bandages and suggested different types of materials to try. Phase I was extended to try and practice these new materials and methods.

Impact on the Study: There are no adverse affects on the study nor is the integrity of the study compromised.

Change Number 3:

Change: In Amendment 1 under change 1 for the paragraph in section 10. Objectives: on page 1 of MREF Protocol 108, delete the last sentence of the paragraph and insert the following sentence:

The ability of the test articles to protect the wound and/or promote wound healing is to be evaluated relative to a no treatment control and to each other.

Reasons for Change: The sponsor modified the study design to resolve study issues, simplify the study, reduce study variables encountered during method development, and align the study to meet the sponsor's needs.

Impact on the Study: There are no adverse affects on the study nor is the integrity of the study compromised.

Change Number 4:

Change: Delete the following sentence of section 11. B. 1. b. Test Material on page 4 of MREF Protocol 108.

The control article is a TWD that may include, but is not limited to, cadaveric human skin (obtained through a tissue bank) or swine skin.

Insert the following sentence to section 11. B. 1. b. Test Material on page 4 of MREF Protocol 108.

The control article is an untreated site.

Reasons for Changes: On October 16 and 17, 1996 the method used to produce HD-induced lesions, issues arising from conduct of phase 1, and study results were presented and demonstrated to the sponsor. The sponsor modified the study design to resolve study issues, simplify the study, reduce study variables encountered during method development, and align the study to meet the sponsor's needs.

Impact on the Study: There are no adverse affects on the study nor is the integrity of the study compromised. This change simplifies the study.

Change Number 5:

Change: Delete the following paragraph under the Test Material section 11. B. 1. a., on page 4 of MREF Protocol 108 as amended in Amendment 1 Change Number 2.

The test article is Dermatograft-Transitional Covering (DGTC) provided and manufactured by Advanced Tissue Sciences.

Insert the following paragraph under the Test Material section 11. B. 1. a. on page 4 of MREF Protocol 108 as amended in Amendment 1 Change Number 2.

The test articles are Dermatograft-Transitional Covering (DGTC, a TWD) and an autograft. The DGTC is provided and manufactured by Advanced Tissue Sciences. The autograft will be harvested prior to surgical preparation of treatment sites. The procedures and methods for harvesting and applying the skin graft will be determined during Phase I.

Reasons for Change: The sponsor modified the study design to resolve study issues, simplify the study, and reduce study variables encountered during method development and align the study to meet the sponsor's needs.

Impact on the Study: There are no adverse affects on the study nor is the integrity of the study compromised.

Change Number 6:

Change: Delete the word "DGTC" and replace with "test articles" in the following sentence of section 11. D. 2. Test Article Evaluation (Phase II) of MREF Protocol 108 as amended in Amendment 1 Change Number 3 so that it reads as follows:

Unless otherwise specified by the sponsor monitor, two treatment sites for control articles and four treatment sites for test articles per animal are used (a

total of 6 treatment sites per animal).

Reasons for Changes: On October 16 and 17, 1996 the method used to produce HD-induced lesions, issues arising from conduct of phase 1, and study results were presented and demonstrated to the sponsor. The sponsor modified the study design to resolve study issues, simplify the study, reduce study variables encountered during method development, and align the study to meet the sponsor's needs. Autograft and DGTC were to be the treatments and the control sites were not treated.

Impact on the Study: There are no adverse affects on the study nor is the integrity of the study compromised. This change simplifies the study.

Change Number 7:

Change: Add the following sentences to section 11. D.1. b. TWD Implantation on page 7 after the sentence "Once the site is prepared, a TWD is applied to the wound" in the middle of the first paragraph.

Of the six HD-induced lesions, two contralateral sites receive no treatment (such as A and B), two contralateral sites receive the autograft (such as C and D), and two contralateral sites receive DGTC (such as E and F). Each treatment will be applied to each treatment location at least once over a period of dosing (at least four). For instance: no treatment applied to sites A and B for Dose 1, sites C and D for Dose 2, and sites E and F for Dose 3, etc.

Reasons for Changes: On October 16 and 17, 1996 the method used to produce HD-induced lesions, issues arising from conduct of phase 1, and study results were presented and demonstrated to the sponsor. The sponsor modified the study design to resolve study issues, simplify the study, reduce study variables encountered during method development, and align the study to meet the sponsor's needs. Autograft and DGTC were to be the treatments and the control sites were not treated.

Impact on the Study: There are no adverse affects on the study nor is the integrity of the study compromised. This change should simplify the study.

Change Number 8:

Change: Throughout the protocol and four amendments where ever suture is written; add "or other acceptable method of tissue application or wound closure".

Reasons for Change: Alternates methods have been discussed with the sponsor for attaching the TWD and autograft. The study director would like to find the best method for applying these treatments besides suturing.

Impact on the Study: There are no adverse affects on the study nor is the integrity of the study compromised.

Change Number 9:

Change: Throughout the protocol and four amendments where ever reference is made to applying an autograft to the DGTC or TWD, change to read:

This procedure may be included as part of the study at the discretion of the study director after consultation with the sponsor.

Reasons for Change: On October 16 and 17, 1996 the method used to produce HD-induced lesions, trial application of test articles, issues arising from conduct of Phase I, and study results were presented and demonstrated to the sponsor. The sponsor modified the study design to resolve study issues, simplify the study, reduce study variables encountered during method development, and align the study to meet the sponsor's needs. Autograft and DGTC are to be the treatments and the control sites are not treated. Comparisons are to be made between the treatment groups. Adding this additional procedure is thought to complicate the study. The study director and sponsor will evaluate additional animals in Phase I without this process.

Impact on the Study: There are no adverse affects on the study nor is the integrity of the study compromised.

Change Number 10:

Change: Under the Statistical Analyses section 14. on page 14 of MREF Protocol 108 and as amended, where ever the protocol mentions "control treatment" in the second paragraph replace it with "autograft".

Reason for Change: Statistical analyses will occur between each treatment. The sponsor is also interested in if DGTC treatment is as good as or better than an autograft.

Impact of Change: There are no adverse affects on the study nor is the integrity of the study compromised.

Change Number 11:

Change: Under the Statistical Analyses section 14. on page 15 of MREF Protocol 108 and as amended, where ever the protocol mentions "control treatment" in the third sentence of the second paragraph of the Clinical wound healing score and the first sentence of the second paragraph of Wound size add the following:

"and to each other"

Reason for Change: Statistical analyses will occur between each treatment. The sponsor is also interested in if DGTC treatment is as good as or better than an autograft.

Impact of Change: There are no adverse affects on the study nor is the integrity of the study compromised.

Frances M. Reid

Frances M. Reid
Study Director

11-1-96
Date

Richard R. Stotts

LTC Richard R. Stotts, COR
USAMRICD

20 DEC 96
Date

In Vivo Evaluation of Temporary Wound Dressings For Adherence, Durability and Autografting on Sulfur Mustard-Induced Lesions in Weanling Swine

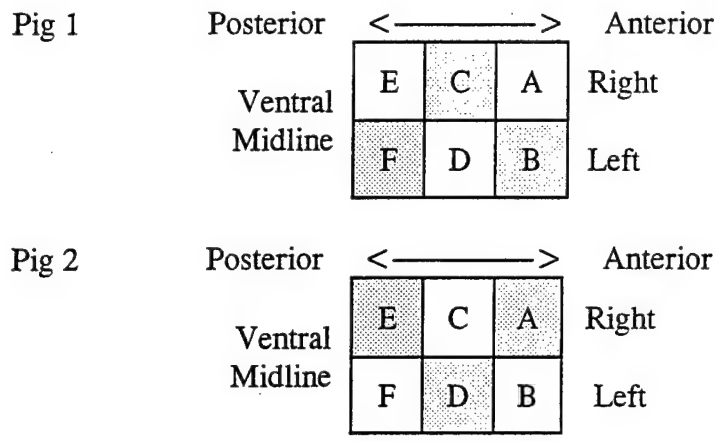
MREF Protocol 108 (G155533A). Amendment No. 6.

Affective Date: December 20, 1996

Addition No. 1: Add after Section 11.D.1.e. (2) on page 10.

"Study Design for the next six animals - Six additional animals are used to characterize HD-induced lesions using histopathology. Histopathological endpoints are to be identified for use in Phase II. The method to produce the lesions consists of using Nair® (applied for seven min), to remove hair from the ventral and lateral thoracic-abdominal area the day before dosing. On the day of dosing, an animal is sedated/anesthetized with a combination of Telazol® and xylazine or other suitable preanesthetic and anesthesia maintained with isoflurane. Once an animal has been anesthetized and three templates per side applied, the animal is placed in a sling within the fume hood for dosing. Four hundred microliters of HD is applied to each dose site, and the site occluded with a Teflon® disc and covered with a rubber stopper. A tile float is placed over the stoppers on each side and secured in place with Vetwrap® for the 2-hr exposure. Once the 2-hr exposure is completed, the animal is decontaminated and placed in a cage within the hood until proof-of-decontamination the next day. On the second day after dosing, the animal is anesthetized again as described above. An approximately 8 mm-sized punch biopsy will be taken from the center of each lesion. A second punch biopsy is taken from the periphery, including normal skin, at a similar location on each lesion. Three lesion sites will be debrided using the method determined by the client. No debridement will be done to the other three sites. Each site will be biopsied twice as described above. The wound treatment grid for two animals, which is to be repeated with the remaining animals, is shown below".

Figure 1. Mustard-Induced Lesions and Sites for Debridement



The shaded areas are lesions to be debrided.

Measurements to be taken:

A. Histopathological evaluations will be made for:

1) Depth of lesion - a quantitative score is to be assigned according to the depth of the lesion. Measurements are made from some point (such as the dermal-subcuticular junction) upward towards the epidermis. A numerical scale is needed for statistical purposes. Each biopsy site will be evaluated for depth of lesion (approximately 72 samples).

2) Wound severity - a quantitative score determined by the pathologist, is assigned to the center biopsy punches of the non-debrided sites only (approximately 18 samples).

B. Clinical pathology analysis will be conducted for:

1) A minimum of 8 mL of whole blood from a single draw is collected from each animal. Duplicate samples may be taken. The sample will be divided for the following analyses:

a. A minimum of 1 mL whole blood is placed into EDTA tubes and mixed to prevent blood coagulation. Samples will be analyzed by Larry Marsh or other Battelle clinical pathology staff for complete blood cell counts (CBC) and differential white cell counts (white blood cell count, neutrophils, lymphocytes, monocytes,

eosinophils, basophils, red blood cells, hemoglobin, hematocrit, mean cell hemoglobin concentration, and platelets).

b. A minimum of 2 mL whole blood is placed into EDTA tubes and mixed to prevent blood coagulation. The sample is centrifuged, the plasma pulled off , and the remaining red blood cells frozen for shipment to MRICD for HD hemoglobin adduct assay analysis. Samples are stored frozen until all samples have been collected and then sent to USAMRICD on dry ice.

c. The remainder of the blood is placed in serum separator-tubes for collection of serum. This sample will be centrifuged after clotting and the serum poured off and frozen. Once all samples have been collected, they will be shipped on dry ice to USAMRICD for analysis. Samples will be prepared in duplicate.

Blood samples will be collected about the same time period (+ or - 30 min) each day for each animal, except when additional samples may be required. For example, 0900 blood sample collected first for animal No.1. The remainder of the blood samples for that animal on different days will be collected approximately +/- 30 min of 0900. A blood sample may be collected for baseline values when the animal is anesthetized for hair removal, and then during preparation prior to dosing, after dosing, after removal from the hood, and daily thereafter for up to nine days post-dosing or as determined by the Study Director in consultation with the COR.

2. Urine sample collection will be attempted while the animals are anesthetized for blood collection. The urine collected is split into two samples, one for urinalysis and one for measuring thiodiglycol. Urine samples are immediately frozen and later shipped on dry ice to USAMRICD.

a) Routine Urinalysis - Duplicate samples are frozen immediately and shipped on dry ice to USAMRICD once all samples are collected.

b) Thiodiglycol analysis - Samples are frozen immediately and shipped on dry ice to USAMRICD once all samples are collected.

Once sample collection is complete for each animal, the pig may be euthanatized. Training in dermatome techniques for obtaining consistent, uniform split thickness tissue may be conducted on these animals prior to euthanasia. Maintenance of these animals may occur for several days after the dermatome procedure for training in the care of sites and bandaging techniques.

Statistics:

Histopathology and clinical score will be made at each site for each animal. The histopathology endpoints will include a measure of skin thickness. An analysis of variance model will be fitted to quantitative data to assess the effects of animal, site location, and possibly debridement techniques on the measured data. In addition, the variability of the response at debrided and non-debrided site will be computed for selected quantitative endpoints.

Reason for Addition: The sponsor requested this additional scope be incorporated in this study.

Impact on Study: This additional information will enhance the value of this study to the client.

Addition No.2. To Section 11.E. Add:

"6. Urine collection maybe requested by the sponsor and will be appropriately accomplished inconsultation with management and the staff veterinarian".

Reason for Addition: To accommodate the sponsor's request for urine collection and analysis during the course of this study.

Impact on Study: None

Deletion No.1: Delete Amendment 2. Pages 25 thru 37.

Reason for Deletion: The original scoring regimen for clinical observations and historical evaluations of wound sites, was not adequate to fully describe the nature of the developing wounds. Therefore, an updated scoring regimen will be developed.

Impact on Study: The new scoring regimen will provide a better study design.

Deletion No. 2: Deleting Amendment No.3, change 9, 10, 11, 12 and 13 (pages 40 thru 42).

Reason for Deletion: These changes concern Amendment No.2, which is being deleted from the protocol.

Impact on Study: None

Frances M Reid

Frances M. Reid
Study Director

12-20-96

Date

Richard R Stotts

LTC Richard R. Stotts, COR
USAMRICD

20 DEC 96

Date

An In Vivo Evaluation of Temporary Wound Dressings For Adherence, Durability and Autografting on Sulfur Mustard-Induced Lesions in Weanling Swine

MREF Protocol 108: Amendment No. 7

Effective Date: January 29, 1997

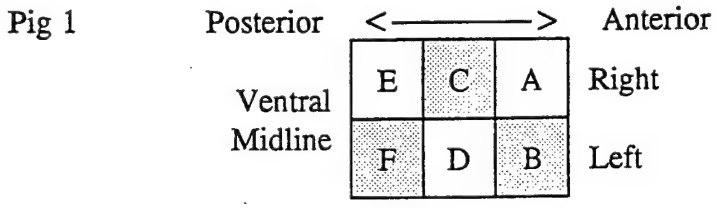
PHASE I FEASIBILITY STUDY

THE STUDY DESIGN IS AS FOLLOWS FOR SIX ADDITIONAL ANIMALS:

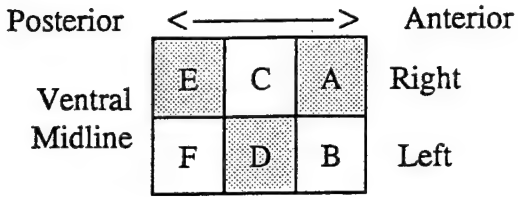
Six additional swine for characterizing debrided and non-debrided HD-induced wounds have been approved for use in Phase I of Task 94-33. These serve as controls for Phase II experiments.

Study Design - Six additional animals are used to characterize HD-induced lesions on non-debrided and debrided wounds using histopathology. Pathological endpoints are to be identified for use in Phase II. The method to produce the lesions using a two hr exposure of 400 microliters of HD applied to a 3-cm diameter Whatman® No. 2 glass microfiber filter template placed on the animal's ventral abdomen is described below. On the second day after dosing, the animal is anesthetized again as described under Wound Development. Three lesion sites are debrided using a method determined by the client (such as dermatome at an approximate 0.75 mm setting). No debridement is performed on the other three sites. After debridement, each site is biopsied twice. An approximately 8 mm-sized punch biopsy is taken from the center of each lesion. A second punch biopsy is taken from the periphery, including normal skin, at a similar location on each lesion. The wound treatment grid for two animals, which is to be repeated with the remaining animals, is shown below.

Figure 1. Mustard-Induced Lesions and Sites for Debridement



Pig 2



The shaded areas are lesions to be debrided.

Measurements taken: Samples may be prepared in duplicate (both histopathology slides and clinical pathology samples).

A. Histopathological evaluations are made for:

1) Depth of lesion - a quantitative score is to be assigned according to the depth of the lesion. Measurements are made from some point (such as the dermal-subcuticular junction) upward towards the epidermis as determined by the pathologist. A numerical scale is needed for statistical purposes. Each biopsy site is evaluated for depth of lesion (approximately 72 samples).

2) Wound severity - a quantitative score determined by the pathologist, is assigned to the center biopsy punches of the non-debrided sites only (approximately 18 samples). Scoring definitions and an example for this histopathological evaluation are attached.

Areas on the slides that are read by Battelle's pathologist are marked by an indelible marker so that the same site can be evaluated by USAMRICD personnel. Once the slides have been read by Battelle's pathologist, these slides are shipped, periodically, to appropriate staff at USAMRICD.

B. Clinical pathology analyses are to be conducted.

1) A minimum of 4 mL of whole blood from a single draw is collected from each animal at or about a targeted time each day of collection. Sample dates and times are to be determined by the Study Director in consultation with the COR and Sponsor. The sample is divided for the following analyses:

a. A minimum of 1 mL whole blood is placed into an EDTA tube and mixed

to prevent blood coagulation. Samples are analyzed by Larry Marsh or other Battelle clinical pathology staff for complete blood cell counts (CBC, which includes but is not limited to: red blood cell count, white blood cell count, hematocrit, hemoglobin concentration, mean corpuscular volume, mean cell hemoglobin concentration, mean corpuscular hemoglobin, and platelets) and differential white cell counts (Diff, includes but is not limited to: neutrophils, lymphocytes, monocytes, eosinophils, and basophils).

b. The remainder of the blood is placed into a serum separator tube for collection of serum. This sample is centrifuged after the blood has clotted and the serum poured off and frozen at approximately - 70 degrees C. Samples maintained frozen and shipped on dry ice to USAMRICD for analysis (at periodic intervals).

Blood samples are collected about the same time period (+ or - 30 min) each day of collection for each animal, except when additional samples may be required or procedures being conducted during the study interfere (such as: animal is being maintained in the hood, proof of decontamination, or surgery). For example, 0900 blood sample collected first for animal 1. The remainder of the blood samples for that animal on different days are collected approximately +/- 30 min of 0900. Blood samples are collected while the animal is awake. However, if attempts to collect blood on an awake animal fail, then the animal is anesthetized to draw blood. Baseline samples are collected prior to dosing. An example of samples collected may be as follows: When the animal is handled for hair removal or during preparation prior to dosing, after removal from the hood (while anesthetized), 24 hr , 72 hr, and 7 days after exposure or as determined by the Study Director in consultation with the COR.

2. Urine samples to measure the total volume of urine over a maximum of approximately 12 hr intervals are collected using housing systems modified to collect urine and/or house swine. At the time of collection, the urine may be filtered, the total volume measured, and a homogenized minimum 5 mL sample drawn for routine urinalysis (specific graviety, glucose, bilirubin, ketone, pH, protein, urobilinogen, blood, nitrite, and leukocytes at a minimum) and thiodiglycol analyses. The urine sample is immediately frozen, and maintained frozen at approximately - 70 degrees C, and later shipped on dry ice to USAMRICD for thiodiglycol analyses (at periodic intervals). The remainder of the urine sample may be discarded after data and sample has been collected.

Mr. John Graham supplied information on the clinical pathology samples to be collected and directions on how to ship samples and this has been incorporated into this plan.

Once sample collection is complete for each animal, the pig may be euthanatized. Training in dermatome techniques for obtaining consistent, uniform split-thickness tissue may be conducted on these animals prior to euthanasia. These animals may be maintained for several days after the dermatome procedure for training in the care of sites and bandaging techniques.

Statistics:

Histopathology and clinical scores are made of each site for each animal. The histopathology endpoints include a measure of skin thickness. An analysis of variance model is fitted to quantitative data to assess the effects of animal, site location, and possibly debridement techniques on the measured data. In addition, the variability of the response at debrided and non-debrided site is computed for selected quantitative endpoints.

Frances M. Reid — 1-30-97

Frances M. Reid, D.V.M.
Study Director

Date

Richard R. Stotts — 30 JAN 97

LTC Richard R. Stotts, COR
USAMRICD

Date

In Vivo Evaluation of Temporary Wound Dressings For Adherence, Durability and Autografting on Sulfur Mustard-Induced Lesions in Weanling Swine

MREF Protocol 108. Amendment No. 8

Change Number 1:

Change: On page 2, 11. A. 2. Age and Weight of the first sentense; delete 10 and replace with 6.

Reasons for Change: The weight range for animals used on this study were determined to be lower than originally thought. Smaller animals were needed to enable handling of animals within the fume hood system. Larger animals, greater than 30 lbs, do not fit into the modified cage in the hood.

Impact on the Study: There are no adverse affects on the study nor is the integrity of the study compromised. An ideal weight for this model is about 18 lbs at dosing.

Frances M. Reid
Frances M. Reid
Study Director

2-19-97
Date

Richard K Stotts
LTC Richard R. Stotts, COR
USAMRICD

21 Feb 97
Date

In Vivo Evaluation of Temporary Wound Dressings For Adherence, Durability and Autografting on Sulfur Mustard-Induced Lesions in Weanling Swine

MREF Protocol 108. Amendment No. 9

Change Number 1:

Add the following procedure for the conduct of Phase II.

Phase II will use 24 animals to evaluate the efficacy of Dermagraft-TC and autografts on healing sulfur mustard-induced full-thickness or deep-partial burns. Nair® applied to the ventral abdomen and lateral sides the afternoon prior to dosing. On dosing day, the animal is anesthetized, templates applied and dosed as described in the last 12 animals (400 μL HD applied to a Whatman Nos. 2 microfiber glass filter for 2 hr exposure, decontaminated and placed in a cage within the hood, and removed approximately 24 hours after dosing and proof of decontamination). There are six sites per animal, divided into 3 treatment groups: a no treatment group, an autograft group, and Dermagraft-TC group. The dose sites for each group will be contralateral. Each group is rotated over the dose sites (similar to those animals dosed in the first Phase I extension of six animals). Small circular tattoo dots are placed at each of the four corners of each dose site for reference either during template application or Day 2 prior to application of grafts.

On Day 2, the deepest setting of the dermatome will be used to dermatome the autograft and Dermagraft-TC application sites. LTC Janny recommended to begin the dermatome cut several mm out from the periphery of the lesion so that we're at our deepest cut when we are at the lesion and we make a square cut of the area. We want a flat area. Staple outside of lesion area on normal skin. Use only a few staples to secure. BE SURE THAT AIR BUBBLES AND BLOOD CLOTS ARE OUT FROM UNDERNEATH THE GRAFT SITES AND GRAFTS ARE FIRMLY AGAINST THE LESION. In bandaging the sites, we need to use Xeroform® or Vasiline impergnated guaze wadded directly over the entire lesion. Mineral oil soaked gauze is uniformly pressed down over the entire grafted lesion area. Dry gauze is used on the no treatment site. Multiple gauze pads are used to provide even pressure over the grafts. Tongue depressors or alternate gauze support may be used. Tongue Depresssors are usually not long enough or wide enough to provide even pressure. An alternate method is to to make a plaster of paris mold or use a plastic mold to hold bandages in place. Secure 4X8 telfa® bandage using Skin Bond®. The animal may be wrapped in Vetwrap® and a stockinette to secure bandages. Maintain bandaging for approximately 14 days. Change bandages on Monday, Wednesday, and Friday, except for the animal who has had surgery on Thursday before Friday's bandage change.

Maintain animals for 38 days. Euthanatize and remove lesions for histopathology evaluations. Clinical observation will be on Day 2 then every Friday there after. On Day 38, when the animals are euthanized, clinical evaluations and excision of the dose sites are taken for histopathology.

For histopathology, there will be two types of scoring. One for wound development on Day 2 and one for wound healing on Day 38. Dr. Singer will provide input on how to score these wounds while we provide him with categories of interest. The categories identified for histologic evaluation of wound severity for Day 2 lesions may be, but are not limited to the following: epidermal necrosis, follicular necrosis, dermal necrosis, dermal neutrophil infiltration, dermal edema, subcutaneous hemorrhage, subcutaneous neutrophil infiltration, subcutaneous edema, and adnexal necrosis. Categories identified for wound healing may be, but are not limited to the following: thickness of the epidermis, amount of vascularization, amount of granulation, and presence of adnexal structures. Wound development and wound healing scoring will be determined.

Frances M. Reid — 3-6-97

Frances M. Reid
Study Director

Date

Richard R. Stotts — 6 MAR 97

LTC Richard R. Stotts, COR
USAMRICD

Date

In Vivo Evaluation of Temporary Wound Dressings For Adherence, Durability and Autografting on Sulfur Mustard-Induced Lesions in Weanling Swine

MREF Protocol 108. Amendment 10.

Effective Date: August 10, 1998

Addition: Add Phase III as follows:

Introduction

Task 94-33 developed full-thickness skin burns on each of six ventral abdominal sites to test a temporary wound dressing (Dermagraft-TC™). Sulfur mustard (HD) was applied percutaneously to each of 6 sites on the ventral abdomen at a dose volume of 400 μL for a 2 hr exposure to produce full-thickness burns. On day 2 (approximately 48 hr after HD application), four of the lesions were excised using a dermatome at 1 mm depth and an autograft (2 sites) or Dermagraft-TC™ (2 sites) applied. During the Iran-Iraq conflict, Kadivar and Adams reported that HD casualties received hospital treatment by day 2.[1] It is estimated that it would take between 48 and 96 hours to deliver an exposed soldier from the battlefield arena to a properly-equipped treatment facility. Therefore, day 2 post-exposure will be the focus for therapeutic regimens and pathophysiology of cutaneous lesions in this burn model.

The histopathologic definition for a full-thickness dermal burn used in this study includes the dermal structures and may involve the panniculus muscle. The histopathologic definition for a superficial dermal burn used in this study includes the epidermis and the upper third of the dermis as defined by Arturson.[2]

A burn encompassing 20 per cent or greater of the body surface area is reported to be considered life threatening by researchers of Institute of Surgical Research in San Antonio. In the current weanling swine model, the burns cover less than 20 percent of the body surface area. The current model would require modifications to wound size, HD concentration, and application techniques to assess safety and efficacy of therapeutic regimens designed for wounds covering greater than 20 percent of the body surface area. Phase III is designed to:

A. Evaluate various solvents used as diluents for HD percutaneous exposure to include stability testing,

B. Choose an anesthetic regimen with minimal effects or consistent vascular effects on

physiological endpoints [degree of erythema, bloodflow (microcirculation) and transdermal water loss] for each dermal dose site using specified equipment, (Minolta Chromameter, Laser Doppler Perfusion Imager, and Evaporimeter, respectively).

C. Characterize the full-thickness of superficial dermal HD-induced wounds, using current dose volume and technique by varying exposure time, and by using dermal monitoring equipment (Laser Doppler, Ultrasound, Evaporimeter, and Minolta® Chromometer), immunohistochemistry, and histopathology with special staining techniques,

D. Determine if full-thickness and superficial dermal burns can be developed, using the current dose volume and technique, by using the maximum allowable concentration of HD (calculated to cover a 20 percent body surface area for an approximately 10 kg animal) in a suitable diluent applied percutaneously and characterize these lesions using equipment, immunohistochemistry, and histology as described in Part C above.

These wounds will be characterized using the evaluation methods described below.

Purpose:

Part A. Solubility, Stability, and Hydrolysis Testing:

The purpose is to identify an appropriate sulfur mustard diluent for cutaneous application using solubility, stability, and hydrolysis testing.

Part B. Anesthesia Effects

The purpose is to choose an anesthetic regimen with minimal effects or consistent vascular effects on dermal dose sites using specified equipment (see section Bioengineering Evaluation beginning on page 73 for equipment descriptions). Three anesthetic regimens are to be tested in six animals using a cross-over design with a one-week washout between treatments. Each site is evaluated at least once using Laser Doppler, Minolta Chromameter, Evaporimeter, and possible alternate analysis systems. These evaluations are expected to identify an anesthetic regimen to be used in subsequent HD-induced skin burn studies. The anesthetic regimen that is identified in Part B will be used in Parts C and D, and subsequent experiments using this model, primarily during clinical observations and data collection.

Part C. Development of Full-thickness and Superficial Dermal Sulfur Mustard-induced Burns by Varying the Exposure Time in Weanling Swine.

The purpose is to develop and characterize full-thickness and superficial dermal burns in weanling swine. Using the current model's HD dosing technique and dose volume, this study will determine if a full-thickness and superficial dermal burn can be produced by varying the exposure time to neat HD in anesthetized weanling swine. Each wound will be characterized using specified equipment (described on page 73 in the Bioengineering Evaluation section), clinical evaluations, and histopathologic and immunohistochemical endpoints. Each wound is evaluated using Ultrasound, Laser Doppler, Minolta Chromameter, Evaporimeter, and possible alternate analysis equipment.

Part D. Determine if Full-thickness and Superficial Dermal Sulfur Mustard-induced Burns can be Produced at a Dilution Calculated to Cover 20 Percent of the Body Surface Area in Weanling Swine.

The purpose is to determine if a dilution of HD, calculated to cover a minimum of 20 percent body surface area and within a maximum of 4 hr exposure time, can produce a full-thickness and/or superficial dermal wound area on the ventral abdomen in weanling swine. These wounds will be characterized using histopathologic and immunohistochemical techniques. Each wound will be characterized using specified equipment (described on page 73 in the Bioengineering Evaluation section), clinical evaluations, and histopathologic and immunohistochemical endpoints. Each wound is evaluated using Ultrasound, Laser Doppler, Minolta Chromameter, Evaporimeter, and possible alternate analysis equipment

Experimental Design for Part A

A minimum of four diluents are to be evaluated. The following diluents are recommended: polyethylene glycol (PEG 200, 400, and 800), peanut oil, propylene glycol, and a possible alternate diluent or combination diluent. The Whatman No .2 glass micro fiber filter paper will need to be evaluated for binding to the diluents. A literature search will be performed to identify possible alternate diluents that exhibit low volatility and minimal to no toxicity by percutaneous absorption. Stability endpoint should be at least 14 days. Dilutions of HD will be 25, 50, and 75 percent solutions, but others may be added upon request. Stability samples will be maintained at room temperature and if considerable degradation is observed, then samples will be stored at approximately minus 70 degrees F.

Experimental Design for Part B

Six female weanling Yorkshire pigs (6 ventral sites per pig) will be used to evaluate three anesthetic regimens using a cross-over design with a one-week washout between treatments. Such as: Each of the three forms of anesthesia are tested on two animals each week, as follows:

Animal	Period Anesthesia	Period Anesthesia	Period Anesthesia
1	A	B	C
2	A	C	B
3	B	A	C
4	B	C	A
5	C	A	B
6	C	B	A

In the table above, A, B, and C, are used to indicate the 3 anesthesia regimens. Regimen A is to use a preanesthetic (such as: 0.044 mg/mL of the mixture of Telazol® reconstituted with a 100 mg xylazine/mL solution) for initiating anesthesia and Isoflurane inhalation anesthesia to maintain anesthesia. Regimen B is to use Isoflurane inhalant anesthesia only. The animal may be masked down or an alternate method of Isoflurane administration developed to reduce stress. Regimen C is to use a combination of Xylazine and Telazol® in repeated injections to maintain anesthesia.

Two animals from each treatment group will be evaluated over a 2 week period and at least two animals evaluated per day of experiment. The following is a recommended schedule: In week one, evaluations may begin with 2 animals on Monday and 2 animals on Tuesday. In week two, during the washout period for the first four animals, the last 2 animals are evaluated. In week three, the treatments are rotated and the animals re-evaluated in the order of original evaluation. This schedule repeats itself until all treatments have been rotated through each animal.

The dosing area will be marked using the plastic template with tatoo marks placed at the corners and evaluations made approximately in the center of each dose site. Evaluations will begin approximately 5-10 min after the anesthesia has been administered (depending on the anesthetic regimen used) and preparatory procedures have been completed. Once each site has been evaluated a second evaluation may be made following the same order as long as no animal is

maintained under anesthesia for greater than 2 hours not including recovery time. The time is noted for beginning of each reading for each site per piece of equipment. The animals will be evaluated for approximately 2 hr every other day for 5 days. Each wound is evaluated using Laser Doppler, Minolta Chromameter, Evaporimeter, and possible alternate analysis equipment (in this order). The equipment is used to make quantitative assessments of blood flow (microcirculation), degree of erhythema, and transdermal water loss, respectively of HD-induced wounds. These evaluations are expected to identify an anesthetic regimen to be used during baseline and wound evaluation data collection in subsequent HD-induced skin burn studies.

Experimental Design for Parts C and D

Agent Exposure: No agent exposure will be conducted in Part B.

Part A: HD will be used in solubility studies.

Part C and D: A minimum of 2, but a maximum of six female weanling Yorkshire pigs (6 ventral sites per pig) may be used in a pilot study to examine the depth of burns induced by varying lengths of exposure to 400 μL HD applied percutaneously. These exposures will use dosing templates and procedures used in Phase II of Task 33, to include the template construction and placement; and placement of Teflon disks, rubber stoppers, and tile floats over the dosing templates following HD application. Exposure time will be varied over a range of 120 min with 20 min intervals in the first animal. Exposure times may be adjusted for the second animal based on the first animal's histopathology results. Repeated exposure times are rotated anterior to posterior to prevent an anterior to posterior bias. Left side versus right side bias is not being considered in this study. Animals will be maintained until study day 2 (study day 0 being the day of HD exposure). Animals are euthanized on day 2 after evaluations are made. Clinical observations, gross photography, high frequency ultrasound, reflectance colorimetry, Laser Doppler perfusion imaging, and evaporimetry will be conducted for each site on each animal. Evaluations are made prior to HD exposure and on day 2 prior to euthanasia. Histopathology of dermal samples on the first animal will be evaluated prior to dosing the next animal. Histopathology will confirm depth and severity of lesions for each site and identify exposure times that induced the depth of lesions required for proper evaluation of treatments for full-thickness burn defined in the Histology section below.

Six animals will be used for sham exposures or diluent controls and treated exactly the same as the above animals, except HD will not be applied to templates. Three control animals for each depth of wound group, will be treated for the maximum time of each group. Evaluations and data collection are the same as described in the paragraph above. These animals will be equally divided between depth of wound groups (full-thickness and superficial dermal burns) and will be

evenly distributed through the dosing regimen for each group. These animals will serve as the study controls. An additional control site near the dosing sites on the ventral abdomen of HD exposed animals may be added to determine if percutaneous HD exposure affects control sites placed on the same animal. If significant differences are observed within and between study animal sham/control sites and the six control animals for parameters measured, then the data for exposed sites will be compared to that of the control animals.

Once exposure times for each wound depth (full-thickness and superficial dermal) are identified, 6 female weanling Yorkshire pigs (6 ventral sites per pig) per wound depth will be exposed to a percutaneous 400 μL HD dose volume per site (6 sites) for the exposure time determined for each wound depth (full-thickness or superficial dermal burn). Animals will be euthanized on day 2 post-HD exposure. Clinical observations, gross photography, high frequency ultrasound, reflectance colorimetry, Laser Doppler perfusion imaging, evaporimetry, clinical pathology, immunohistochemistry, and histopathology will be conducted for each animal. Clinical and equipment evaluations, and photographs will be taken before dosing and on study day 2 before euthanasia.

Statistics: For the pilot animals, dosing times will be arranged to minimize the effect of anterior/posterior differences and animal to animal variability. This is accomplished by mixing the longer and shorter dosing times over both animals and over sites A, B, E, and F on each animal. For example, if 10 minute increments are selected, then the following scheme is used.

Sites		Animal 1 Dose Times (min)		Animal 2 Dose Times (min)	
A	B	90	30	120	20
C	D	50	70	80	60
E	F	10	110	40	100

Dosing times on the second animal may be adjusted based on the first animal's histopathology results.

Histopathology: Tissue samples will be excised on study day 2 after euthanasia. Detailed discussions of the evaluations and histopathology are described in their respective sections below. An exposure time is identified when a full-thickness or superficial dermal burn is suspected to consistently occur at 48 hr post-exposure in 80 percent of the animals within a set. This exposure time for the specified depth of burn will be used for future studies using this model.

Part C: The concentration of HD used will be neat HD.

Part D: The concentration of HD used will be a dilution determined to cover 20 percent or greater of the body surface area of the pig. The diluent will be determined in Part A and the concentration the maximum allowed to cover the area by chemical surety and safety constraints. The exposure times for this dilution will not exceed 4 hr. The first animal challenged will include the two hour exposure time. The second animal may have the exposures time increased to a maximum of four hours, if the first animal's histopathology indicates that the full-thickness and/or superficial dermal burn have not been produced.

ANALGESIA

Experience has shown that weanling pigs cutaneously exposed to neat HD for 2 hr display some signs of discomfort (e.g., attempts to scratch at the exposure sites) which may correlate with the degree of redness and amount of edema present in these lesions starting at 4 hr post-exposure. The vasoactive peptide bradykinin, whose production is catalyzed by activated Hageman factor during the inflammatory response, is a potent pain producing agent whose effect is potentiated by prostaglandins. Due to the extent of the edema formation in these severe HD lesions, it is possible that these inflammatory mediators are causing some degree of discomfort in these animals. Pain and severe itching sensations have been noted in humans during the development of cutaneous HD lesions.[1,3-6] Therefore, an analgesic such as buprenorphine (0.005-0.01 mg/kg IM.) will be used on a consistent, routine basis. For this part of Phase III, the analgesic will be administered (1) immediately after exposure and before the animal is placed in the holding cage in the exposure hood, and (2) early the following morning on day 1. No analgesic administration should be necessary on day 2. Antibiotics or anti-inflammatories will not be used during this phase of the task. Additional animals may be required to replace animals removed from the study requiring the use of antibiotics or anitinflammatory drugs.

Measurements/Observations

Clinical observations (see Clinical Observation Evaluation Work Sheet beginning on page 86 for parameters measured) photography, high frequency ultrasound, reflectance colorimetry, Laser Doppler perfusion imaging, evaporimetry, and clinical pathology will be conducted throughout the study except where noted. Other applicable bioengineering methodologies which can be used to quantitatively assess wound development may also be utilized.

Clinical Observations and Gross Photography

Clinical observations will be conducted on study day 2 using the grading scheme enumerated in

Clinical Observation Evaluation Work Sheet beginning on page 86 (tattooing of the animal may be performed, but measurements for wound contraction will not be made during this part of Phase III). Gross photography will be conducted along with the clinical observations. The three lesions of the same side of the ventral midline will be photographed together with a metric ruler and appropriately identifying labeling within the photograph. The teats and marks delineating the dosing sites will be clearly visible, and the camera angled in such a position that the line of sight is perpendicular to the cutaneous surface, and the camera is at a measured focal distance from the sites (to be consistent for each photograph and specified on clinical observation sheets). Additionally, pictures will be taken of each side at an angle such that the degree of edema (height) can be visualized. These photographs will serve not only as a visual record of the developing lesions, but also allow future morphological measurements via image analysis, if needed. Bioengineering evaluations, using the afore mentioned equipment, will be read before HD exposure in Parts C and D for each pilot and study animals.

BIOENGINEERING EVALUATIONS

High Frequency Ultrasound

High frequency ultrasound (HFU) will be conducted before exposure and on post-exposure study day 2 to measure overall skin thickness. HFU has been utilized in the past to measure increases in skin thickness due to edema formation in the hairless guinea pigs and pigs exposed to HD vapors.[7-9] Ultrasonic pulse-echo techniques have also been used by medical researchers in non-invasive depth determinations of thermal burns.[10-17] During evaluations, HFU evaluations are performed last. HFU will be used in this task to quantitate increases in skin thickness due to accumulation of edema, and to examine its use as a possible non-invasive tool to determine burn depth. An instrument such as the DermaScan C Ultrasonic 3D or 2D skin scanner (Cortex Technology, Denmark) or equivalent will be utilized. Two-dimensional (B-scan) depth profiles of dosing sites will be captured onto a personal computer for permanent storage. The images will be captured while the animal is under anesthesia to minimize imaging problems associated with movement. A layer of ultrasound transmission gel (e.g., Aquasonic® 100, Parker Laboratories, Inc., Orange, NJ) will first be applied over the site to be imaged, and an image captured, and saved to disc on an IBM-compatible personal computer. For images captured before exposure, the hand-held probe will be positioned over the center of the demarcated exposure site. For the images captured on study day 2, two images will be saved. The first image will involve centering the ultrasound head over the edge of the lesion, to visualize any increase in edema from unaffected tissue to affected tissue. No measurements will be made on these edge shots; they will be used for visualization only. The second image will be made over the center of the lesion, on which measurements will be conducted. Images will be oriented vertically such that the epidermis can be seen on the left and subcutaneous tissues on the right. The dermal-subcutaneous junction needs to be clearly visualized (good visualization of the

panniculus muscle will greatly help in this effort, and an attempt should be made to include it in all images). The swept gain set for the left (epidermis) and right (subcutis) surfaces will be recorded. These swept gains should be set during the first ultrasound imaging and remain the same for all images recorded in this phase, if at all possible. HFU software will be used to make the thickness measurements immediately after each image is captured, while the animal is still under anesthesia. In the event that the captured image does not provide adequate material in which to make the measurements, another image of that site will be immediately captured and analyzed. Horizontal lines will be interactively drawn at set positions over each image, setting upper and lower limits for the thickness measurements. Approximately two-thirds of each image will be delineated for measurement. Interactive edge detection will then be performed by setting A-scan thresholds, representing signal reflection amplitudes, to outline both the outer epidermal surface and the dermal/subcutis interface over the entire length of the delineated area. Threshold settings for each edge will be recorded. Distance measurements will then be automatically performed by the software for each A-scan line in the B-scan image and reported as a mean and standard deviation. On a site-by-site basis, the before exposure measurements will be subtracted from the measurements made on study day 2, in order to calculate the change in (Δ) thickness values to be used in statistical analyses.

Reflectance Colorimetry

Sites will be evaluated for degree of erythema using a reflectance color meter (e.g., Chromameter Model CR-300, Minolta Corporation, Ramsey, NJ) before exposure and on study day 2. The Minolta Chromameter uses reflected light and reads color in a three-dimensional format giving the brightness between black and white, the balance between red and green, and the relative amounts of yellow and blue. The unit includes a small hand-held measuring head connected to a portable data-processing unit by a flexible cord. Three photocells measure the light reflected by the surface of the sample (over an area of an 8-mm diameter circle). The detected signal is converted into three coordinates (L*, a*, and b*) of a three-dimensional color system recommended by the Commission Internationale de l'Eclairage (CIE). This color coordinate system most closely represents human sensitivity to color. The coordinate L* represents levels of brightness between white (+100) and black (-100); the a* represents the relative chromaticity between red (+60) and green (-60); and the b* coordinate represents the relative amounts of yellow (+60) and blue (-60). The a* chromaticity parameter will be used in this study as the indicator of the degree of erythema (redness). This coordinate is directly related to the degree of color redness and is the most appropriate parameter to use for evaluating erythema. The chromameter is better able to discriminate close shades of red than the human eye, especially for bright red shades. Reflectance colorimetry has been used in the past to provide a reproducible, objective, and quantitative assessment of vesicant skin injury in both euthymic hairless guinea pigs and weanling pigs.[7-9,18,19] The chromameter is to be calibrated before each session using a white Minolta calibration plate which has been wiped clean with an absorbent alcohol wipe.

Four replicate readings will be taken on each site at the periphery of a site and at a consistent location for each lesion; and four replicate readings will be taken from the center of the lesion. On a site by site basis, the four replicate readings from each location will be averaged, resulting in a single a* value for each site location. The net increase in erythema (Δ a*) following exposure will then be calculated by subtracting the before-HD reading.

Laser Doppler Perfusion Imaging

Laser Doppler perfusion imaging (LDPI) will be utilized to study continuous blood flow (microcirculation). The complex vascular network of the dermis plays a crucial role in thermoregulation, nutrition and metabolism. Laser Doppler flowmetry and LDPI have been used for prolonged, non-invasive monitoring of tissue viability, and for assessment of inflammation, ischemia, wound healing, reperfusion, and burn depth.[20-34] LDPI may prove useful in delineating the areas of damage that need to be debrided, avoiding areas with sufficient blood flow. It also has the potential for being a useful tool in evaluating the effects of pharmacologic agents which decrease edema and/or increase blood flow in the microcirculation, therapies which may be explored in the future. It may also prove useful in the determination of adequate skin graft take. It should be noted that ambient temperature and airflow will need to be controlled and kept relatively constant when this methodology is utilized. Measurements are to be made under controlled conditions, and environmental measurements of temperature and humidity during the testing will be recorded. Measurements will be made as soon after anesthesia as possible, and conducted before HD exposure and on study day 2. Unaffected skin surrounding each site will be incorporated in each image. The day 2 results are to be correlated later with depth of burn as determined by histopathology, in order to ascertain if this methodology can be used as a non-invasive method to determine the depth of the cutaneous HD burn.

Evaporimetry

Finally, stratum corneum barrier function will be tested by measuring transepidermal water loss (evaporimetry) using an instrument such as the Evaporimeter Model EP-2 (Servo Med AB, Sweden). The stratum corneum forms a barrier-against diffusion of wear through the epidermis, and thus also the penetration of various chemicals which come in contact with the skin. The efficiency of this layer depends upon the integrity of the stacking of the corneocytes and their intracellular adhesion, as well as the water content of the layer. Evaporimetry has been used in the testing of cutaneous irritants, and in the evaluation of clinical skin conditions (irritations or diseases) and occlusive pharmaceutical/cosmetic preparations.[35-37] This methodology holds promise as a non-invasive way of determining how soon after agent exposure barrier function has been compromised, and when normal barrier function has returned. **Note that external environmental factors such as room temperature, relative humidity, air turbulence, probe temperature, and pressure application of the probe on the skin will need to be controlled**

and kept relatively constant when this methodology is utilized. Multiple measurements from different, set locations within each lesion will be taken and the results averaged. Transepidermal water loss measurements will also be taken from unaffected skin between each site and the ventral midline. Measurements will be made on study day 0 before HD exposure and on study day 2 as soon after anesthesia is administered as possible.

Excellent overviews of high-frequency ultrasound, reflectance colorimetry, evaporimetry and Laser Doppler flowmetry/perfusion imaging can be found in Jorgen Serup's *Handbook of Non-Invasive Methods and the Skin* (CRC Press, 1995).

Anesthesia

Most anesthetic agents cause cardiovascular depression via hypothermia and effects on myocardium, blood vessels and the nervous system.[37] Anesthesia-induced peripheral vasoconstriction is thus a concern, and could affect the results obtained for reflectance colorimetry, Laser Doppler imaging, and evaporimetry. The regimen of xylazine HCL and equal parts of tiletamine HCl and zolazepam HCl, which has been used in this animal model historically, may result in peripheral vasoconstriction, which may substantially affect these results. The anesthetic regimen that causes the fewest and least severe changes in these parameters will be identified in Part B. The anesthetic regimen identified in Part B will be used to conduct Parts C and D and future studies with this model.

Clinical Pathology

Leukopenia has been reported in human cases of severe systemic intoxication to HD, which may be a result from both direct action of the agent on hematopoietic tissue and a secondary, bacterial infection facilitated by damage to skin and/or respiratory epithelium.[39] Changes in clinical pathology parameters following HD exposure have been noted in both hairless guinea pigs[40] and weanling pigs.[10] As HD is systemically absorbed following cutaneous exposure, hematology and clinical chemistry examinations will be conducted to ascertain the presence of systemic toxicities. Blood samples will be collected just prior to agent exposure, and on day 2 just prior to euthanasia. Eight to ten ml of blood will be taken by venipuncture from the anterior vena cave or other appropriate site at each time point (day 0 and day 2) and immediately after anesthesia is induced. Blood samples will be divided into EDTA tubes and serum separation tubes. The following clinical pathology parameters will be measured: complete blood cell count (CBC), differential white cell counts, hematocrit, hemoglobin, mean cell volume (RBC), mean cell hemoglobin, mean cell hemoglobin concentration, electrolytes (chloride, sodium, potassium, calcium, phosphorus), blood urea nitrogen (BUN), creatinine, BUN:creatinine ratio, glucose (hexokinase), alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase

(ALP), lactate dehydrogenase (LDH), creatine phosphokinase (CPK), amylase, total protein (TP), albumin, globulin and albumin:globulin ratio. Isoenzymes of LDH and CPK will be measured on fresh (never frozen) serum samples using classic electrophoretic techniques. Each pre-exposure sample will serve as the animal's own control reference sample. Each parameter will be evaluated for significant changes using appropriate statistical methods.

Histopathology

Following euthanasia on day 2, each entire exposure site with surrounding normal tissue (full-thickness, and extending ventrally to include the teat line and dorsally to include several cm of normal tissue beyond the lesion) will be excised using a scalpel blade. "Full-thickness" will be defined as including the panniculus muscle. The collection of deep muscle groups will not be necessary (e.g., external abdominal oblique, latissimus dorsi or serratus anterior). The primary lesion will be bisected in a direction parallel to the ventral midline. The biopsy half closest to the ventral midline will be stapled onto an index card to prevent curling, through the corners of the biopsy closest to the ventral midline, and placed into 10% neutral buffered formalin. After adequate fixation time, these tissue samples will be trimmed in a direction perpendicular to the ventral midline such that some surrounding normal skin is included in the section. Each trimmed piece will measure approximately 3.0 cm in length (with approx. 1.0 cm of untreated skin and 2.0 cm of HD-damaged skin). An additional, shorter piece of full-thickness skin measuring approx. 1.5 cm long will be trimmed from the untreated area lateral to the treatment site (trimmed parallel to the ventral midline and taken just adjacent to the teat line). Both pieces will be placed in the same tissue processing cassette, processed and embedded in paraffin with the epidermal surfaces of both pieces oriented in the same direction. Sections will be cut and stained with hematoxylin and eosin (H&E) for routine histopathologic evaluation.

The biopsy half closest to the dorsal midline will have strips cut out of it in a direction perpendicular to the ventral midline. Each trimmed piece will be approximately 3-4 mm wide and contain approximately 2-3 mm of untreated skin and approximately 10-12 mm of HD-damaged skin. Three such pieces will be placed on a frozen tissue cassette and surrounded by O.C.T. embedding media. The cassettes will be snap frozen in liquid freon, wrapped in a double layer of aluminum foil, and stored at approximately -70°C until processed. A few pieces of untreated ventral skin will also be collected from each pig and processed in O.C.T. similarly. Frozen sections will be cut at 12 microns (one section from each dosing site). On frozen tissues, the reduction of nitro blue tetrazolium chloride (NBTC) by the cell-bound enzyme nicotinamide adenine dinucleotide diaphorase (NADH-diaphorase) leads to an intensive blue granular precipitate (diformazan granules) at the sites of NADH-diaphorase activity. NADH-diaphorase activity has been demonstrated to subside upon cell death, thus viable (blue) and damaged (unstained) cells can be clearly differentiated.[41,43] NBTC staining will thus be used to help

differentiate viable from non-viable tissue. Directions/training for tissue cryopreservation, frozen sectioning on a cryostat, and NBTC staining will be provided by MRICD employees. Over time, stains on frozen tissue can fade, hence all NBTC-stained sections will be photo documented as the stains are generated. For each section, color Kodachromes (2x2") will be prepared which incorporate the junction between normal and HD-damaged skin. Additionally, Kodachromes will be prepared which cover the center of the HD lesion itself. Several magnifications will be used. At the end of the experiment, all remaining formalin-fixed wet tissues, paraffin blocks and frozen tissue will be shipped to MRICD for possible special staining and immunohistochemistry. The duplicates of gross photographs, H&E- and NBTC-stained sections will be returned to MRICD as well, for possible image analysis.

In addition, kidney and each diaphragmatic lobe of the lung will be collected and half placed in formalin and half in a fixative supplied by USAMRICD. Representatives from USAMRICD will demonstrate what samples are required.

The histopathological evaluations will be conducted by a board-certified veterinary pathologist (diplomate of the American College of Veterinary Pathology) in the employ of Battelle. The evaluation of H&E-stained sections will be as follows:

a. Depth of necrosis

0 = none
1 = squamous epithelium only
2 = follicular structures involved
3 = dermal structures involved
4 = panniculus (adipose and/or carnosa) involved

b. Necrosis of basal epithelial cells within the damaged area

0 = none
- = none visible, but granulation tissue is present, indicating a previous necrotic injury had occurred
1 = < 5% of area involved
2 = 10-40% of area involved
3 = 50-80% of area involved
4 = >90% of area involved

c. Ulceration

0 = absent
1 = present

d. Granulation tissue

0 = none
1 = minimal
2 = mild
3 = moderate
4 = severe

e. Re-epithelialization

0 = none (epithelial defect present with no re-epithelialization)
- = epithelium not yet lost
1 = < 5% of area from wound margin to cut end of section is covered
2 = 10-40% of area from wound margin to cut end of section is covered
3 = 50-80% of area from wound margin to cut end of section is covered
4 = >90% of area from wound margin to cut end of section is covered

Specific grading criteria and numerical rating scales for the NBTC-stained sections will be determined by the pathologist after a general review of the slides. A general understanding for the depth of non-viable tissue present in each section is desired.

The results from evaluation of the H&E- and NBTC-stained sections are to be combined for each exposure site, and the overall "depth of burn" characterized. If possible, lesions are to be classified as either "superficial", "superficial dermal (partial thickness)", "deep dermal (partial thickness)" or "full thickness". Criteria for these burn depths can be found in John AD Settle's text entitled "Principals and Practice of Burns Management" (Churchill Livingstone, 1996).[2] New definitions describing burn depth may need to be constructed which are more appropriate for HD rather than thermal burns.

Statistics

Appropriate statistical tests (such as descriptive statistics and figures for each piece of equipment, clinical observations and histopathology; and possible ANOVAs for specified

endpoints requested by the client or any trends observed) will be conducted by Battelle on data collected as described below.

Part A - No statistical analysis is planned.

Part B - A 3 phase cross-over design is employed for these experiments to minimize animal usage. The effects of anesthesia on readings from Laser Doppler, Minolta® chromameter, evaporimeter, and possible alternate analysis systems are evaluated statistically. Mean readings under the three forms of anesthesia are compared using analysis of variance (ANOVA) models appropriate for cross-over designs. The assumption of normality for the distribution of readings is assessed visually. If this assumption is grossly violated, then either a transformation will be applied to the data prior to fitting the ANOVA models, or the analysis will be conducted using nonparametric methods.

Parts C and D - Similar statistical methods are applied to data collected in parts C and D. The primary comparison of interest is that between full-thickness and superficial dermal wounds. Additional comparisons are made between HD dosed animals and appropriate control animals.

Statistical analyses will focus on high frequency ultrasound, reflectance colorimetry, Laser Doppler perfusion imaging, and evaporimetry readings. For each instrument, the difference between baseline and day 2 readings is calculated for each site on each animal. Mean differences are compared using ANOVA models. The assumption of normality for the distribution of readings is assessed visually. If this assumption is grossly violated, then either a transformation will be applied to the data prior to fitting the ANOVA models, or the analysis will be conducted using nonparametric methods. In addition, instrument readings may be compared qualitatively or graphically to appropriate clinical or histopathology scores.

Comparisons of clinical observation scores, wound size, and histopathology scores on day 2 are made using ANOVA models or categorical methods. Within each group of HD dosed animals, clinical pathology parameters are compared between baseline and day 2 using paired t-tests. Comparison of the effects of full-thickness and superficial dermal burns on these parameters are made using two-sample t-tests. Alternatively, these comparisons may be made using ANOVA models, or nonparametric methods if the assumption of normality is grossly violated.

Guidelines for Clinical Observations

Attached are the guidelines and rating scales to be used for conducting clinical observations. The following parameters are to be measured/graded at the time points enumerated above: size of wound, exudate, eschar, percent area covered by eschar or scab, extent of erythema, description

of erythema, hemorrhage, edema, extent of necrosis, infection (suppuration), and general health. Sample forms on which to record clinical observations in the animal rooms are also included.

Data Storage

Data collected form clinical observations, high frequency ultrasound, reflectance colorimetry, Laser Doppler perfusion imaging, evaporimetry, clinical pathology, and histopathology are entered into a Microsoft Access database to facilitate data management and the transfer of data to the statistician. If needed, data are transferred to Microsoft Excel spreadsheets for transfer to USAMRICD personnel.

Equipment

Some equipment to be used in this task will be provided by USAMRICD, and some by Battelle, as follows:

USAMRICD will supply the equipment used for evaporimetry and high frequency ultrasound (including transmission gel), and the instruction to utilize those methodologies.

Battelle will provide the equipment used for reflectance colorimetry (Minolta Chromameter Model CR-300, including white calibration plate), gross and microscopic photography (digital camera), clinical pathology, and histopathology.

A moorLDI™ Laser Doppler perfusion imager (Moor Instruments, Inc., Wilmington, DE) is being ordered for USAMRICD. It is anticipated that it may take six months to complete the procurement process. Should the equipment be received before the animal work on this task is scheduled to begin, it will be shipped out to the MREF for use on this study. Should the instrument be delayed in its arrival beyond the start of the animal work, Battelle shall lease the imager from Moor Instruments until such time that USAMRICD's imager is received and shipped out to the MREF. The approximate cost of the lease is $2,700.00 per month (excluding shipping and handling charges), with delivery of the equipment usually within 4 weeks from receipt of the order.

Room temperature and humidity are to be recorded during the performance of all bioengineering methodologies and clinical observations. A digital printing hygrometer/thermometer such as the Thermo-Hygro Recorder Model NEW 11-661-1 7A with an accessory AC adapter, probe and recording paper) is to be provided by Battelle. All bioengineering methodologies should be conducted in the same environmentally controlled room, if possible, with the hygrometer/thermometer probe placed near the work area.

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Clinical Observation Evaluation worksheet

1. Size of wound

a. Length (mm)
b. Breadth (mm)

- calipers will be used to make the measurements
- length = anterior (cranial) to posterior (caudal); 9 to 3 o'clock
- breadth = left to right; 12 to 6 o'clock - measured over the area of erythema or scab, but not the area of edema
- on surgical wounds, the whole wound is measured, not just the HD-dosed area; as the edge of the wound heals (e.g., day 14-21), measure just the remaining scab-covered area (e.g., the periphery of a dermatomed area blends into normal skin over time, and cannot be easily measured)

2. Exudate

0 = absent
1 = present but moist
2 = present as dried scab (e.g., crusty, especially around edges)
* = can't evaluate, due to eschar or other condition

3. Eschar (slough made of several cell layers, usually visible by day 7)

0 = absent
1 = present

4. Percent Area Covered by Eschar or Scab

0 = none
1 = less than 25% of original dosing area involved
2 = at least 25% but less than 50% of original dosing area involved
3 = at least 50% but less than 75% of original dosing area involved
4 = 75% or greater of original dosing area involved

5. Extent of Erythema (pink, red or deep red)

0 = none present
1 = present along border or along border and within border
2 = beyond border, and inclusive of #1
* = not observable, due to scab, eschar or other condition

Note: The comment section can be used for more in-depth descriptions.

6. Description of Erythema (darkest hue present)

0 = none
1 = pink
2 = red
3 = deep red

7. Hemorrhage (purple)

0 = absent
1 = present
* = not observable, due to scab, eschar or other condition

8. Edema (measurements made via calipers)

a. Height (mm above teat line?)
b. Length (mm)
c. Breadth (mm)
d. Visual Score:

0 = none
1 = minimal - barely perceptible or questionable
2 = mild - area raised approximately 1 mm, may have well defined edges
3 = moderate- area raised approximately 2 to 3 mm, well defined and may be spreading
4 = severe - area raised 4 mm or more, and extending beyond area of exposure

9. Extent of Necrosis (white patches)

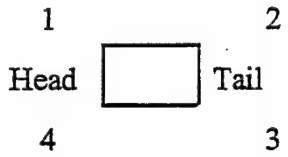
0 = none
1 = less than 25% of original dosing area involved
2 = at least 25% but less than 50% of original dosing area involved
3 = at least 50% but less than 75% of original dosing area involved
4 = 75% or greater of original dosing area involved
* = not 0, but cannot be adequately observed due to coverage by scab, graft, or other condition

10. Infection (suppuration)

0 = absent
1 = present

11. Wound contraction

a. Place a tattoo mark (small "+" or "X") beside each of the two anterior-most dosing sites, and beside each of the two posterior-most dosing sites. The tattoos should be at least 1.5 cm beyond the edge of each of those four 5 x 5 cm delineated sites, and positioned midway between the dorsal and ventral outer corners of the sites. The tattoo marks should be mentally numbered as follows:

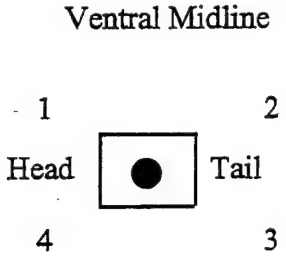


b. The following measurements will be made with a metric ruler (mm) between the centers of the tattoo marks:

1 → 2
2 → 3
3 → 4
4 → 1
1 → 3
2 → 4

These measurements will be used, along with body weight measurements, to judge the overall growth of the animal during the course of the experiment.

c. On day of surgical manipulation (e.g., day 2), place tattoo marks (small +'s or X's) just to the outside of all four corners of each lesion or surgical graft site, in a consistent manner. The tattoo spots are to be mentally numbered as follows:



d. The following measurements will be made with a caliper (mm) between the centers of the tattoo marks on each site:

1 → 3
2 → 4
1 → edge of lesion, along the diagonal
2 → edge of lesion, along the diagonal
3 → edge of lesion, along the diagonal
4 → edge of lesion, along the diagonal

These measurements will be used to ascertain if the wounds have contracted over time.

12. General health

a. weight (kg)
b. rectal and room temperatures (take immediately after induction of anesthesia)
c. respiratory/ENT problems (e.g., sneezing, runny nose)
d. gastrointestinal problems (e.g., diarrhea)
e. skin problems (not related to exposure sites)
f. other

13. Comments

Approvals:

Frances M. Reid — 11-25-98

Frances M. Reid
Study Director — Date

Richard R Stotts — 1 DEC 1998 RRS

LTC Richard R. Stotts, COR
USAMRICD — Date

QA Registered:

Elisha Morrison — 6/4/99

Elisha N. Morrison, M.S.
Senior Quality Assurance Specialist — Date

MEDICAL RESEARCH AND EVALUATION FACILITY

DEVIATION FORM

Study and/or Facility record affected

Study Title: In Vivo Evaluation of temporary wound dressings for adherence, durability and autografting on HD or other vesicant/irritant induced lesions on weanling swine

Study Number(s): G155533A

Record: Animal observation binder 1 Deviation No. (Assigned by QAU): DR-209

Type of Deviation

- ☐ GLP (Section): _____
- ☑ SOP (Number): VII-027-01
- ☐ Protocol (Number): _____
- ☐ Method (Number): _____
- ☐ Other: _____

Date of Deviation(s): 2/23/99

① Animal #: 99-2-9

Description of Deviation: PM animal observation not documented on this date.

Cause of Deviation: Failure to document

Impact of Deviation: None

Corrective Action: CA No. Assigned by QAU (if applicable): NA

Responsible technician advised to document in the future

If deviation is planned, effective date: NA

Deviation form Prepared by/Date: [signature] 2-22-00

Deviation Reviewed and Corrective Action Accepted by/Date: Francis M. Reid 2-22-00
Study Director (if applicable)

Deviation Reviewed and Registered by QAU/Date: [signature] 5/2/00

Form No. MREF Facility-035-01 (Revised 4/6/98)

① added EMS 5/2/00

MEDICAL RESEARCH AND EVALUATION FACILITY

DEVIATION FORM

Study and/or Facility record affected

Study Title: In Vivo Evaluation of temporary wound dressings for adherence, durability and autografting on HD or other vesicant/irritant induced lesions on weanling swine

Study Number(s): G155533A

Record: Study binders 1 and 2 Deviation No. (Assigned by QAU): DR-210

Type of Deviation

☐ GLP (Section): _____

☐ SOP (Number): _____

☒ Protocol (Number): 108

☐ Method (Number): _____

☐ Other: _____

Date of Deviation(s): various dates

Description of Deviation: Several animals were under anesthesia for more than 2 hours

Cause of Deviation: Instrument problems and inability to keep animals anesthetized with certain anesthetic Regimens

Impact of Deviation: It allowed us to determine which anesthetic regimen would be sufficient to use in phase III part c

Corrective Action: Instruments were repaired

CA No. Assigned by QAU (if applicable): NA

If deviation is planned, effective date: NA

Deviation form Prepared by/Date: [signature] 2-28-00

Deviation Reviewed and Corrective Action Accepted by/Date: James W. Reed 2-28-00
Study Director (if applicable)

Deviation Reviewed and Registered by QAU/Date: [signature] 5/2/00

Form No. MREF Facility-035-01 (Revised 4/6/98)

MEDICAL RESEARCH AND EVALUATION FACILITY

DEVIATION FORM

Study and/or Facility record affected

Study Title: In Vivo Evaluation of temporary wound dressings for adherence, durability and autografting on HD or other vesicant/irritant induced lesions on weanling swine

Study Number(s): G155533A

Record: Animal observation binder 1 Deviation No. (Assigned by QAU): DR-21

Type of Deviation

- ☐ GLP (Section): _____
- ☐ SOP (Number): _____
- ☑ Protocol (Number): 108
- ☐ Method (Number): _____
- ☐ Other: _____

Date of Deviation(s): 2/16/99, 3/4/99, 3/23/99, 3/25/99, 3/29/99

Description of Deviation: Record of animal fasting not documented

Cause of Deviation: Failure to document

Impact of Deviation: None, fasting occurred but not documented

Corrective Action: CA No. Assigned by QAU (if applicable): NA

Responsible technicians advised to document in the future

If deviation is planned, effective date: NA

Deviation form Prepared by/Date: [signature] 2-22-00

Deviation Reviewed and Corrective Action Accepted by/Date: [signature] 2-22-00
Study Director (if applicable)

Deviation Reviewed and Registered by QAU/Date: [signature] 5/3/00

Form No. MREF Facility-035-01 (Revised 4/6/98)

MEDICAL RESEARCH AND EVALUATION FACILITY

DEVIATION FORM

Study and/or Facility record affected

Study Title: In Vivo Evaluation of temporary wound dressings for adherence, durability and autografting on HD or other vesicant/irritant induced lesions in weanling swine

Study Number(s): G155533A

Record: Study binder phase III part C Deviation No. (Assigned by QAU): DR-212

Type of Deviation

☐ GLP (Section): _____

☐ SOP (Number): _____

☒ Protocol (Number): 108

☐ Method (Number): _____

☐ Other: _____

Date of Deviation(s): 7/26/99

(1) Animal # 99-70-10

Description of Deviation: Missing weight record

Cause of Deviation: Weight record misplaced

Impact of Deviation: None, the animal was weighed the day before study and all anesthetics were prepared accordingly

Corrective Action: CA No. Assigned by QAU (if applicable): NA

The lead technician will be careful to see that all paperwork will be placed in the study record binder as soon as possible

If deviation is planned, effective date: NA

Deviation form Prepared by/Date: [signature] 4-25-00

Deviation Reviewed and Corrective Action Accepted by/Date: [signature] 4-25-00
Study Director (if applicable)

Deviation Reviewed and Registered by QAU/Date: [signature] 5/3/00

(1) added Emw 5/3/00

ATTACHMENT B

Phase I Letter Report Dated November 18, 1997

For Review and Approval

Project No. G1555-9RDM

Internal Distribution

FM Reid
CT Olson
RMO
File

	Name	Initials	Date
Originator	FM Reid	FMR	11-18-97
Concurrence	CT Olson w/ changes	CTO	11/18/97
Approved	JB Johnson	JBJ	11/18/97

November 18, 1997

LTC Richard R. Stotts, VC, COR
Battelle Columbus Operations
505 King Avenue, Building JM-3
Columbus, OH 43201-2693

Dear LTC Stotts:

Contract No. DAMD17-89-C-9050
Letter Report on Task 94-33

This letter report is on the first 16 animals of Phase I of the Medical Research and Evaluation Facility (MREF) Task 94-33, "*In Vivo* Evaluation of Temporary Wound Dressings For Adherence, Durability and Autografting on Sulfur Mustard-Induced Lesions in Weanling Swine". The first phase was a feasibility study to determine if a deep partial- or full-thickness sulfur mustard-induced burn could be developed consistently in weanling swine.

BACKGROUND

The U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) is responsible for evaluating treatment regimens for percutaneous bis (2-chloroethyl) sulfide (sulfur mustard; HD) exposure and for developing models to make these assessments. HD is bifunctional in that it is an alkylating agent and also can produce incapacitating vesicant injuries. Swine are reported to exhibit dermal pathology similar to that observed in humans. This phase of the study was designed to develop consistent deep partial- to full-thickness HD-induced skin lesions in weanling pigs.

The domestic pig has been reported to have morphologic and functional skin characteristics comparable to human skin.[1] These common characteristics include: 1) sparse-hair coat, 2) a thick epidermis with distinct rete pegs and corresponding dermal papillae, 3) a relatively high concentration of elastic fibers in the dermis, 4) hair follicles with similar vascularity, 5) similar collagenous tissue framework and adipose chambers in the subcutis, 6) comparable enzyme patterns in the epidermis with similar apocrine glands, 7) analagous epidermal tissue turnover time and keratinous protein character, and 8) similar composition of the lipid film of the skin surface.[1] There are also, however, differences. The following characteristics of domestic pig skin differ from those of human skin: 1) vascularity of the cutaneous glands and subepidermal plexus is poor, 2) eccrine glands are absent in the common integument, 3) extensive fat is deposited below the

subcutis, 4) there is a positive alkaline phosphatase reaction in the basal stratum of the epidermis, 5) a negative or slightly positive reaction for hydrolytic enzymes (particularly alkaline phosphatase) occurs in the capillaries of the subepidermal vascular plexus, 6) strong enzyme reactions occur in active connective tissue cells of the dermis, 7) seasonal shedding of hair exists, and 8) apocrine skin glands are not involved in thermoregulation.[1]

The pig is the model of choice for percutaneous HD exposures since it has limited hair cover, fixed skin similar to humans, and exhibits microvesication or epidermal-dermal separation.[2,3,4,5,6,7] In this study, a variety of pretreatments and HD volume combinations were tested to determine a regimen that would consistently produce deep partial- or full-thickness skin burns.

EXPERIMENTAL DESIGN:

Sixteen, female, Yorkshire swine from Isler Genetics, Inc (Prospect, Ohio) were used in a step-wise approach to develop an animal model for deep partial- or full-thickness HD-induced skin burns. Isler Genetics, Inc. is a specific pathogen free (SPF) facility and an animal source approved by Battelle's Attending Veterinarian.

Animals were quarantined upon receipt for a week, during which general physical examinations were performed by a staff veterinarian. Each animal was ear tagged with a number composed of the year of birth, litter number, and pig number. Feed was provided twice a day. Animals were weaned from the swine producer's ration to a laboratory swine grower ration manufactured by PMI Feeds, Inc (St. Louis, MO). For animals being anesthetized, the feed was removed a minimum of 8 hours before anesthetizing. Following anesthesia, feed was gradually increased over several days until full ration was resumed. Water was supplied from the Battelle, West Jefferson water system, and was given *ad libitum*. No contaminants that would affect the results of the study are known to be present in the feed or water.

Once exposed, animals were individually housed in mobile, large animal enclosures manufactured by Britz-Heidbrink, Inc (Wheatland, WY). Animals were maintained under fluorescent lighting with a light/dark cycle of approximately 12 hr each per day. Air temperature in swine rooms was maintained between approximately 50 and 80 degrees F. The room temperature was elevated to the upper end of the range at receipt of weanling swine and gradually reduced over time. Relative humidity in animal rooms was maintained between approximately 30 and 70 percent.

Animals at Battelle are cared for in accordance with the guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" (National Academy of Sciences, 1996), and/or in the regulations and standards as promulgated by the Agricultural Research Service, United States Department of Agriculture (USDA), pursuant to the Laboratory Animal Welfare Act of August 24, 1966, as amended. On January 31, 1978, Battelle's Columbus Division received full accreditation of its animal care program and facilities from the American Association for Accreditation of Laboratory Animal Care (AAALAC). Battelle's full accreditation status has been

renewed after every inspection since the original accreditation. The MREF is a part of the facilities granted full accreditation.

General Daily Experiment Preparation:

Six sites on each animal were dosed. Table 1 summarizes the pretreatments and HD volume and exposure time for each animal and each site. Pretreatments were selected to facilitate hair removal, keratin layer removal, or to assist HD penetration into the epidermis. The table of Appendix A presents a detailed summary of HD volume and exposure times, pretreatment compounds and/or techniques used, treatments, and bandaging technique used on each site of each animal. Appendix B describes the procedures used to develop a deep partial- to full-thickness HD skin burn model and a description of results for each animal.

Pre-dosing Preparation: Within 24 hr of dosing, each animal was weighed. The first 16 swine used in Phase I weighed between 7 and 19 kg. In an earlier study, clipping of the coarse hair over dose sites frequently left scratches in the dosing area, and erythema could be observed for at least 24 hr after clipping. A chemical depilatory compound (Nair® AG, Carter Products, Division of Carter-Wallace, Inc., NY, NY) applied to the dosing area for approximately 7 min before rinsing created minimal to no observable irritation the next day. Removal of pretreatment products (Nair®, enzyme therapy, etc) consisted of gentle washing with a 1:20 Ivory® dish-washing detergent (Proctor and Gamble, Cincinnati, Ohio) diluted with distilled water, patting the area with warm water-soaked 4x4 gauze sponges (The Kendall Company Hospital Products, Boston, MA), and patting the area dry with gauze sponges or a clean towel. Enzyme products (trypsin or papain) were generally applied the day prior to dosing, and removed with the mild soap solution before dosing as described above. The second animal had trypsin and papain (Meijer, Grand Rapids, MI) applied to the right and left side of the dosing area, respectively, and then was covered with a stockinette (Alba Health® Non-sterile Stockinette, Health Products Division, Alba-Waldensian, Inc. Rockwood, TN); washing before dosing was accomplished as described above. Animals two through eleven had trypsin (Trypzyme-V®, Veterinary Products Laboratory, Phoenix, AZ) applied to the designated dosing areas and sites were then covered with a stockinette. On the first animal, trypsin was applied to the right dorsum for approximately 2 hr on the day of dosing. Tape stripping was accomplished on the left dorsum prior to dosing. Trypsin was removed, as described above, before dosing with HD.

Table 1. Pretreatment and Dosing Summary for Sixteen Animals Used to Develop Sulfur Mustard-Induced Chemical Burns.

Animal Number	Animal ID	HD Dose[1] (µL-hr)	Right Side Pretreatments									Left Side Pretreatments								
			Site	Nair[2]	Trypsin[3]	Papain[4]	Tape Strip	Sand paper	Emery Paper	Needle	Defat	Site	Nair[2]	Trypsin[3]	Papain[4]	Tape Strip	Sand paper	Emory Paper	Needle	Defat
1	96-13-9[5]	200-2 (A&B)	B	x	x							A	x			x				
		200-1.5(C&D)	D	x	x							C	x			x				
		200-1 (E&F)	F	x	x							E	x			x				
2	96-13-7	500-2	A	x	x					x		B	x		x				x	
			C	x	x			x				D	x		x		x			
			E	x	x							F	x		x					
3 & 4	96-21-7 96-21-10	300-2	A	x	x					x		B	x	x					x	x
			C	x	x			x				D	x	x			x			x
			E	x	x				x			F	x	x				x		x
5	96-34-5	300-2 (A&D)	A	x	x							B	x	x		x				
		500-2 (B&C)	C	x	x							D	x	x		x				
		600-2 (E&F)	E	x	x							F	x	x		x				
6	96-34-4	300-2	A	x	x							B	x	x						
			C	x	x							D	x	x						
			E	x	x							F	x	x						
7-10	96-1-4 96-1-3 96-3-9 96-2-10	400-2	A	x	x							B	x	x						
			C	x	x							D	x	x						
			E	x	x							F	x	x						

Table 1. Pretreatment and Dosing Summary for First Sixteen Animals Used to Develop Sulfur Mustard-Induced Chemical Burns (continued).

Animal Number	Animal ID	HD Dose[1] (μL-hr)	Right Side Pretreatments									Left Side Pretreatments								
			Site	Nair[2]	Trypsin[3]	Papain[4]	Tape Strip	Sand paper	Emery Paper	Needle	Defat	Site	Nair[2]	Trypsin[3]	Papain[4]	Tape Strip	Sand paper	Emory Paper	Needle	Defat
11	96-5-8	400-2	A	x	x							B	x							
			C	x	x							D	x							
			E	x	x							F	x							
12-15	96-10-12 96-10-11 96-11-8 96-11-11	400-2	A	x								B	x							
			C	x								D	x							
			E	x								F	x							
16	96-18-8	400-2	A	x								B	x							
			C	x								D	x							
			E	x								F	x							

[1] All HD doses were applied for 2 hours, except for animal 96-13-9. On this animal, sites A & B were dosed for 2 hrs, C & D were dosed for 1.5 hours, and E & F were dosed for 1 hour.
[2] Nair was generally applied for 7 minutes. Occasionally, two applications were necessary for complete hair removal.
[3] Trypsin was applied overnight, except for animal 96-13-9. Trypsin was applied to sites for 2 hours on this animal.
[4] Papain was applied overnight.
[5] Animal 96-13-19 was dosed on the dorsum. All other animals were dosed on the ventral abdomen.

Dosing Day Preparation (Day 0): Enzyme pretreatments were removed the day of dosing prior to drawing the dosing grid, except for the first animal dosed. Animals were anesthetized for pretreatments, HD exposure, wound excision and/or biopsy, and treatment application (autograft and Dermagraft-TC application). Atropine sulfate (AMVET Scientific Products, Yaphank, NY) was administered intramuscularly (IM) at 0.04 mg/kg body weight (BW) to control excessive salivation. Animals were anesthetized with a combination of xylazine (Ben Venue Laboratories, Inc., Bedford, OH) and Telazol® (Fort Dodge Laboratories. Inc, Fort Dodge, Iowa). Five mL of xylazine solution (100 mg/mL) were used to reconstitute Telazol® (250 mg tiletamine and 250 mg zolazepam) and the mixture dosed at 0.044 mL/kg BW IM. Tracheal intubation was performed and inhalation anesthesia was initiated using a concentration of 2.5 to 3 percent Isoflurane (Abbott Laboratories, North Chicago, IL) in oxygen at an initial flow rate of 2 L per min using an Anesco anesthetic machine (Anesco, Inc, Georgetown, KY) or Matrix anesthetic machine (Matrix Medical Inc., Orchard Park, NY). Anesthesia was maintained with an Isoflurane concentration of approximately 1-1.5 percent with flow rates reduced to a range of 800 mL to 1 L/min. Pretreatments were accomplished and/or dosing templates were applied once the animal was anesthetized. Following HD exposures or surgical application of grafts, the concentration of Isoflurane was reduced over time until the animal was on 100 percent oxygen, and then gradually changed to room air.

Pretreatment Techniques: Appendix A summarizes the various pretreatment techniques, HD dose volumes and exposure times, abrading techniques, and bandaging for each animal. The keratin layer of the skin was stripped away to facilitate HD penetration into the epidermis in order to produce a deep partial- to full- skin thickness burn. Techniques used were tape stripping, abrading, or enzyme treatment. For tape stripping, a section of duct tape (3M, St. Paul, MN) was used to form a loop, adhesive side out, encircling the applicator's fingers, and was applied once in a rolling motion over the designated dose site. For abrading dose sites, a 25 ga hypodermic needle (Becton Dickinson Company, Rutherford, NJ), 3M scouring pad, sand paper (#100, Sears and Roebuck Company, Chicago, IL), or emery paper (3M) was used once over the designated dose site. Enzyme therapy was with trypsin or papain, and was used in combination with some of the abrading techniques. Defatting of the skin was performed just before application of dosing templates or abrading, using an isopropyl alcohol-soaked gauze dabbed on the dose area. As the model was developed, a decision was made to minimize the preparations before dosing, and techniques except for hair removal were eliminated.

A six-site dosing grid (3 sites per side) was drawn on the dorsum or ventral abdomen of each animal, as shown below.

Posterior <———> Anterior

Midline

E	C	A
F	D	B

Each site within the grid measured approximately 5 cm by 5 cm. An approximately 0.5-1 cm space separated each dosing square. For dorsal dose sites, with the pig in ventral recumbency, the upper edge of the dosing grid was drawn parallel to and approximately 1.5 cm lateral to the dorsal

spinal processes on both sides of the animal. For ventral dose sites, with the animal in dorsal recumbency, the dosing grid was drawn approximately 1.5 cm lateral to and parallel to the teat line on both sides of the animal. Dosing templates were applied to each of the six sites prior to placing the animal in a sling within a chemical fume hood. The animal was secured in the sling and allowed to stabilize for up to 20 minutes prior to dosing.

Each dosing template was constructed of a 5 by 5 cm square of duct tape applied to double-sided carpet tape of the same size. A 3-cm diameter circle was cut through the tape assembly, and between the two tapes a Whatman No. 2, microfiber glass filter paper (Whatman. Hillsboro, OR) was placed to cover the 3-cm diameter hole. A 3-cm diameter O-ring (Hewlett Packard, Wilmington, DE) was glued to the outer surface of the tape assembly and surrounded the 3-cm diameter hole.

HD Dosing and Decontamination: Sulfur mustard was supplied by USAMRICD. Purity, appropriate identification and storage condition information was provided by USAMRICD. HD samples were taken and evaluated by the MREF chemistry section to assure concentration. Appendix C contains chemistry analysis data. Samples were analyzed in triplicate by gas chromatography using a flame ionization detector (FID). In each case, the percent of the expected concentration was well within the acceptable ± 20 percent range. Values ranged from 91.1 to 99.5 percent of expected.

HD exposure times (1, 1.5 and 2 hr) and dosing volumes (200, 300, 400, 500 and 600 μL) were varied to determine conditions for producing deep partial- to full-skin thickness HD burns. HD was applied to the 3-cm diameter microfiber filter within the dosing template using either a 250 or 500-μL glass Hamilton syringe (Hamilton Company, Reno, NV) with a blunt 18 ga needle. After application of HD, a size 3 teflon disc (Thomas Scientific Company, Swedesboro, NJ) was placed over the dosing site and a rubber stopper inserted in the dosing well to occlude the dose site, minimize HD evaporation, and achieve a uniform HD application. After several animals were dosed, the rubber stoppers were secured on each side using a rubber tile float taped in place using Vetwrap ® (3M).

After exposures, the animal was decontaminated by gently patting each dosed site with a 2 by 3-cm absorbent swab attached to a tongue depressor to remove excess HD. Absorbent swabs soaked with 1.5 mL of a 0.5 percent sodium hypochlorite solution were then applied to each dose site for 10 sec. Next, 1.5 mL of water was dabbed on each site for 10 sec using 2 by 3 cm absorbent swabs, and this was repeated a second time. After dose site decontamination, a 0.5 percent sodium hypochlorite-soaked sports towel (Musslinn ® Sports Towel, Johnson and Johnson Advanced Material Company, New Brunswick, NJ) was placed over the entire dose grid and covered with a plastic-backed absorbent paper for 4 min. This was followed by 3 water rinses using water-soaked towels applied for 2 min. The dosed sites were allowed to air dry for approximately 15 min prior to placing an approximately 2-L plastic bag over the entire dosed area, and a relatively air-tight seal was formed using double-sided carpet tape cut in lengths to secure the bag to the area. A Minicam® (CSM Company, Birmingham, AL) was used to sample the air within this plastic bag after allowing it to equilibrate for approximately 15 min. The decontamination procedure was altered several times to attain acceptably low HD levels (≤ 0.5

TWA) before removing the animal from the hood. The first two animals were maintained in the hood for approximately 8 hours prior to their removal. The decontamination procedure was then altered: dry gauze sponge was gently pressed against each dose site for 30 sec to remove excess HD. A water-soaked gauze sponge was then gently pressed to each dose site for 30 sec. A water-soaked sports towel was placed over each side of the animal and a plastic-backed absorbent paper sheet was placed over the sports towel and held in place for 1 min. This step was repeated and then the area allowed to air dry for approximately 15 min. The animal was allowed to recover from anesthesia as described earlier, removed from the sling, extubated, and kept in the fume hood overnight in a modified animal transport crate. The next morning, the animal was anesthetized with the Telazol®/xylazine combination and decontamination evaluated using a Minicam. Once proof of decontamination was obtained, the animal was removed from the fume hood and returned to its home cage.

RESULTS:

The results of statistical analyses are presented in Appendix D. Histopathologic and clinical observation data summaries for each animal are presented in Appendix E. The statistical analyses were performed on data from the last five animals, whose burns were full-skin thickness and produced with the following method. Nair® was applied to the dosing area for 7 min the day prior to HD dosing, removing the Nair® with a mild soap solution. On the day of dosing, the animal was anesthetized, dosing templates were applied, and the animal positioned within the fume hood. Four hundred μL of HD was applied to each site for 2 hr, and the animal then decontaminated and kept within a transport cage in the fume hood overnight. After proof-of-decontamination the following day, the anesthetized animal was removed from the fume hood and placed into its cage. Table 2 summarizes the study days on which wounds on these animals were evaluated.

Table 2. Summary of Study Days on Which Histopathologic Evaluations and Clinical Observations Were Made for Animals Pretreated with Nair® and Dosed with 400 µL HD for 2 Hours.

	Histopathologic Evaluation (Study Day)						Clinical Observations (Study Day)							
Animal ID	2	3	6	9	16	23	2	3	6	9	16	23	30	37
96-5-8*		x	x					x	x					
96-10-11	x						x							
96-10-12	x						x							
96-11-8	x				x		x				x**			
96-11-11	x				x		x							
96-18-8	x			x	x	x	x			x	x	x	x	x

* Three sites on this animal received the Nair® only pretreatment.

** Clinical observations were collected on day 16, however the endpoints examined were not consistent with those used for animal 96-18-8 on day 16.

STATISTICAL SUMMARY: Due to the exploratory nature of these experiments, the statistical analysis focused on summarizing Day 2 results for the pretreatment and HD dose volume combination that was selected for use in future phases of this task. The last five animals (96-10-12, 96-10-11, 96-11-8, 96-11-11, and 96-18-8) were pretreated only with Nair® and dosed with 400 μL HD for 2 hours on all six sites. In addition, one other animal (96-5-8) received this combination on the left side (sites B, D, and F). The statistical report on clinical observation and histopathology data collected for MREF Task 94-33, Phase I (First 16 Animals) is presented in Appendix D.

HISTOPATHOLOGY SUMMARY: Table 3 presents the percent incidence of histopathology endpoints on sites pretreated only with Nair® and dosed with 400 μL HD for 2 hr.

Table 3. **Incidence of Histopathology Endpoints on Sites Pretreated with Nair® and Dosed with 400 µL HD for Two Hours.**

Histopathology Endpoint	Incidence [Number Observed / Number Examined (%)]					
	Nair Only Pretreatment					
	Day 2	Day 3	Day 6	Day 9	Day 16	Day 23
Epidermal Necrosis	28/30(93.3)	3/3(100.0)	3/3(100.0)	1/3(33.3)	0/12(0.0)	N/A
Follicular Necrosis	18/30(60.0)	3/3(100.0)	3/3(100.0)	1/3(33.3)	0/12(0.0)	N/A
Epidermal Ulceration	1/30(3.3)	0/3(0.0)	0/3(0.0)	4/6(66.7)	2/13(15.4)	6/6(100.0)
Epidermal Neutrophil Infiltration	1/30(3.3)	0/3(0.0)	2/3(66.7)	4/6(66.7)	0/13(0.0)	5/6(83.3)
Microblister	0/30(0.0)	0/3(0.0)	0/3(0.0)	0/6(0.0)	0/13(0.0)	0/6(0.0)
Slough	1/30(3.3)	0/3(0.0)	0/3(0.0)	5/6(83.3)	8/13(61.5)	6/6(100.0)
Dermal Hemorrhage	0/30(0.0)	1/3(33.3)	0/3(0.0)	2/6(33.3)	0/13(0.0)	2/6(33.3)
Dermal Necrosis	9/30(30.0)	3/3(100.0)	3/3(100.0)	3/6(50.0)	1/13(7.7)	0/6(0.0)
Dermal Ulceration	0/30(0.0)	0/3(0.0)	0/3(0.0)	1/6(16.7)	0/13(0.0)	6/6(100.0)
Dermal Neutrophil Infiltration	22/30(73.3)	3/3(100.0)	3/3(100.0)	6/6(100.0)	12/13(92.3)	5/6(83.3)
Dermal Edema	5/30(16.7)	0/3(0.0)	0/3(0.0)	3/6(50.0)	1/13(7.7)	0/6(0.0)
Neovascularization	0/30(0.0)	0/3(0.0)	0/3(0.0)	3/6(50.0)	2/13(15.4)	0/6(0.0)
Granulation	0/30(0.0)	0/3(0.0)	1/3(33.3)	6/6(100.0)	13/13(100.0)	6/6(100.0)
Subcutaneous Hemorrhage	15/30(50.0)	1/3(33.3)	0/3(0.0)	0/6(0.0)	0/13(0.0)	2/6(33.3)
Subcutaneous Edema	23/30(76.7)	1/3(33.3)	0/3(0.0)	0/6(0.0)	0/13(0.0)	0/6(0.0)
Subcutaneous Neutrophil Infiltration	22/30(73.3)	2/3(66.7)	3/3(100.0)	6/6(100.0)	12/13(92.3)	5/6(83.3)
Subcutaneous Necrosis	2/30(6.7)	3/3(100.0)	3/3(100.0)	3/6(50.0)	7/13(53.8)	0/6(0.0)
Re-epithelialization	0/30(0.0)	0/3(0.0)	0/3(0.0)	5/6(83.3)	7/13(53.8)	6/6(100.0)
Infection	0/30(0.0)	0/3(0.0)	0/3(0.0)	0/6(0.0)	0/13(0.0)	0/6(0.0)

Microblisters and infection were pathologic endpoints not seen in these animals. In addition, dermal hemorrhage was observed infrequently. The following conditions were observed in at least half the sites (n=30) on study day 2: epidermal necrosis (93.3 percent), follicular necrosis (60.0 percent), dermal neutrophil infiltration (73.3 percent), subcutaneous hemorrhage (50.0 percent), subcutaneous edema (76.7 percent) and subcutaneous neutrophil infiltration (73.3 percent). Although fewer sites were examined on later study days, high incidences of dermal neutrophil infiltration and subcutaneous neutrophil infiltration were observed through day 23. Epidermal necrosis and follicular necrosis continued to be evident through study day 6, but not later, and subcutaneous hemorrhage and subcutaneous edema were not observed after study day 3, with the exception that subcutaneous hemorrhage was observed at two sites on day 23. These two hemorrhage sites occurred after the wounds appeared to have healed. Two conditions were observed with high incidence on the middle study days: dermal necrosis on days 3-9 and subcutaneous necrosis on days 3-16. Incidence of dermal edema and neovascularization peaked on study day 9, with each present in 3 of the 6 sites examined on that day. The remaining histopathologic indicators were more often present on later study days. Granulation, re-epithelialization, and sloughing were observed mostly on days 9-23; epidermal ulceration and epidermal neutrophil infiltration were observed on days 9 and 23, but not on day 16. Epidermal neutrophil infiltration also was observed in 2 of 3 sites on day 6.

A more complete summary of histopathologic evaluations is presented in Appendix D, Tables A-1 through A-4 of Addendum A of the Statistics Report. These tables present incidence of histopathologic endpoints on study day two and later for all pretreatment and HD dose combinations where two or more sites were treated.

CLINICAL OBSERVATIONS SUMMARY: Descriptive statistics for clinical observations on the same animals are presented in Table 4 of the statistics report (Appendix D).

Exudate, erytherma, edema, and necrosis were evaluated on days 2, 3, and 6. A clinical wound severity score (WSS) was calculated as the sum of the pathology. Erythema, necrosis, and edema were present at all sites on study day 2, except one site on animal 96-10-11. Erythema scores tended to be most severe, comprising approximately one-half of the WSS. Exudate was observed infrequently, except on animal 96-18-8 where it was observed in 4 of 6 sites. When observed, exudate was mild. Figure 1 of the Statistics Report in Appendix D presents the mean WSS for each animal, overlaid on the observed values for each site. From this figure, it is apparent that the WSS varied considerably, both between sites on an animal and between animals.

Wound size (WS) was measured at each site on each study day. Wound size data are presented in Figures 2 and 3 of the Statistics Report. Figure 2 illustrates the mean WS for each animal on study day 2, overlaid on the observed values for each site. Figure 3 displays the mean WS plotted against time for animal 96-18-8, overlaid on the observed values for each site. On study day 2, WS appears to be fairly consistent between sites on an animal and between animals. For animal 96-18-8, WS is greatest on study day 9, and variability appears to increase with time. However, WS measurements on day 30 appear to be inconsistent with measurements on days 23 and 37. This is attributed to differences in the judgement of individuals taking the measurements, rather than rapid changes in the wounds during this period.

To evaluate wound healing, indications of exudate, inflammation, granulation, contraction, infection, vascularization, and epithelialization were recorded on study days 9, 16, 23, 30, and 37 for animal 96-18-8. Figure 4 of Appendix D presents the mean score for each wound healing parameter, averaged over the 6 sites on animal 96-18-8, plotted against time. The wound healing scores tended to decrease over time, with the exception that inflammation increased through study day 23 and was, in general, not observed thereafter. In addition, granulation scores were higher on day 30 than at earlier times. Granulation was not scored on study day 37 as the wounds appeared to be completely healed.

Analysis of variance (ANOVA) models were fitted to the WSS and WS data collected on study day 2 to assess the animal-to-animal variability and to determine whether there were significant differences among sites. Appropriate contrasts were used to assess whether there were differences between anterior and posterior sites. The ANOVA model took the following form:

$$Y_{ij} = \mu - \alpha_i - \gamma_j - \epsilon_{ij}$$

where Y_{ij} is the observed WSS or WS for site I on animal j, μ is the average WSS or WS, α_i is a fixed site effect, γ_j is a random animal effect, and ϵ_{ij} is a random error term. The Statistical Analysis System (SAS ver. 6.12, Cary, NC) GLM procedure was used to fit the ANOVA models.

No significant effects were detected in the analysis of WS. There was significant animal-to-animal variability in WSS ($p<0.001$, $\sigma_{animal}=1.6$). The overall site effect for WSS was not statistically significant ($p=0.217$), when each site was evaluated individually in the ANOVA model. A more specific test comparing the averages for sites A and B to the averages for sites E and F determined that WSSs were significantly greater on posterior sites than anterior sites ($p=0.025$, estimated difference in means [=SE] = 3.1 [=1.3]). The anterior site versus posterior site test is more sensitive than the overall ANOVA test in detecting the difference. These results are not contradictory. It is sometimes the case that noise in the data may cloud an effect in the overall test, but that a specific effect of interest may be detected by a more specific hypothesis test.

CONCLUSIONS

The regimen used for development of deep partial- or full-skin thickness HD burns started with the use of a chemical depilatory (Nair®) applied for 7 min to the ventral abdomen the day prior to dosing and removal by gentle rinsing using a 1:20 dilution of Ivory dish-washing liquid. On the dosing day (approximately 18 hr later), the animal was anesthetized, a dosing template adhered, the animal secured in a sling in a stanchion within a fume hood, and 400 μL of HD applied to the glass microfiber filter paper of each dosing template. After a 2-hr exposure, the dose sites were decontaminated, and the animal held in a holding cage within the fume hood overnight. The following day, Day 1, after proof of decontamination, the animal was removed from the hood. Day 2 was chosen to debride damaged sites based on articles describing casualty wounds and

treatments from the Iran-Iraq war and USAMRICD directions for development of therapies.[3] Histopathologic endpoints that were consistently reported on Day 2 evaluations were epidermal necrosis, follicular necrosis, dermal neutrophil infiltration, subcutaneous hemorrhage, subcutaneous edema, and subcutaneous neutrophil infiltration.

Clinical observations within and between animals were more variable than histopathologic endpoints. Erythema scores tended to be the most severe. Efforts to combine individual clinical observation scores to create an overall wound score resulted in the erythema accounting for approximately half of the score. It is recommended that clinical observations be evaluated independently and not combined. As new technologies develop, quantitative methods for measuring clinical endpoints should be evaluated to reduce variability in the data. Variability between individuals was noted with clinical observations. Maintaining the same evaluator throughout a study is recommended. The anterior wounds appeared more severe than the posterior wounds, however this was not statistically significant. Treatments need to be rotated through each site to reduce any bias.

Wound size measurements were consistent, particularly on Day 2 evaluations, and variability increased with time. Much of the variability was the result of different evaluators.

Wound healing scores tended to decrease over time. This does not mean that healing was not occurring. This indicated that as the wound healed, the same scoring technique was confusing, and as a result, two separate evaluations were created. The first evaluation was for wound development and emphasized wound severity and wound depth. The second evaluation was for wound healing and emphasized evaluating the amount of granulation tissue, neovascularization, epithelialization, presence of infection, wound size, and wound contraction. These evaluations will be used with the last 6 animals of Phase I and with Phase II animals.

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Please feel free to contact me at (614) 424-7832 or Dr. John B. Johnson at (614) 424-5259 if you need additional information.

Sincerely,

Frances M. Reid

Frances M. Reid, D.V.M.

FMR/cah

cc: LTC James A. Romano, Deputy Director, MCBDRP, USAMRMC
LTC Michelle Ross, Chief, Drug Assessment Div., USAMRICD
Mr. John Blische, AMLO, USAMRICD (ATTN: Melissa Kerns)

APPENDIX A

SUMMARY OF TECHNIQUES USED TO PRODUCE PARTIAL- TO FULL-THICKNESS SKIN BURNS ON EACH ANIMAL

PRETREATMENT, TREATMENT AND BANDAGING TECHNIQUES USED IN PHASE I

KEY

Pretreatment:
N = Nair
TR = Trypsin
TS = Tape Stripping
PA = Papain
SP = Sandpaper
ESP = Emery Sandpaper
NE = Needle
DF = Defat Skin

Treatment (TX):
SE = Surgical Excision
DM = Dermatome
NT = No Treatment
AU = Autograft
TWD = Dermagraft-TC

Bandaging:
TH = Thermazine
NTH = No Thermazine
XG = Xeroform Gauze
VG = Vaseline Gauze
DR = Dry Gauze
SG = Single gauze folded
DG = Double gauze folded
TD1 = Single tongue depressor
TD2 = Two tongue depressors tapped together
NTD = No Tongue depressors used
AST = 4X8 Adhesive Bandage With Surgical Tape
TB = Tissue Bond
BE = Benzoin
VW = Vet Wrap
S = Stockinette
NA = NOT APPLICABLE
MG = Wad of gauze
FLD = Folded gauze

DATE	STUDY DAY	ANIMAL ID	SITE ID DORSAL	HD DOSE uL-hr	PRETX	ABRADE	TX	ANTIBIOT	GAUZE WOUND	GAUZE PACK	TONGUE DEPRES.	TISSUE ADHES.	OTHER BAND.
4/16/96	6	96-13-9	A	200-2	N,TS	No	NA	NA	NA	NA	NA	NA	NA
			B	200-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			C	200-1.5	N,TS	No	NA	NA	NA	NA	NA	NA	NA
			D	200-1.5	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			E	200-1	N,TS	No	NA	NA	NA	NA	NA	NA	NA
			F	200-1	N,TR	No	NA	NA	NA	NA	NA	NA	NA

DATE	STUDY DAY	ANIMAL ID	SITE ID VENTRAL	HD DOSE uL-hr	PRETX	ABRADE	TX	ANTIBIOT	GAUZE WOUND	GAUZE PACK	TONGUE DEPRES.	TISSUE ADHES.	OTHER BAND.
4/29/96	5	96-13-7	A	500-2	N,TR	NE	NA	NA	NA	NA	NA	NA	NA
			B	500-2	N,PA	NE	NA	NA	NA	NA	NA	NA	NA
			B2	500-2	N,PA	NE	NA	NA	NA	NA	NA	NA	NA
			C	500-2	N,TR	SP	NA	NA	NA	NA	NA	NA	NA
			D	500-2	N,PA	SP	NA	NA	NA	NA	NA	NA	NA
			E	500-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			F	500-2	N,PA	No	NA	NA	NA	NA	NA	NA	NA

DATE	STUDY DAY	ANIMAL ID	SITE ID VENTRAL	HD DOSE uL-hr	PRETX	ABRADE	TX	ANTIBIOT	GAUZE WOUND	GAUZE PACK	TONGUE DEPRES.	TISSUE ADHES.	OTHER BAND.
5/9/96	3	96-21-7	A	300-2	N,TR	NE	NA	NA	NA	NA	NA	NA	NA
			B	300-2	N,TR,DF	NE	NA	NA	NA	NA	NA	NA	NA
			C	300-2	N,TR	SP	NA	NA	NA	NA	NA	NA	NA
			D	300-2	N,TR,DF	SP	NA	NA	NA	NA	NA	NA	NA
			E	300-2	N,TR	ESP	NA	NA	NA	NA	NA	NA	NA
			F	300-2	N,TR,DF	ESP	NA	NA	NA	NA	NA	NA	NA

DATE	STUDY DAY	ANIMAL ID	SITE ID VENTRAL	HD DOSE uL-hr	PRETX	ABRADE	TX	ANTIBIOT	GAUZE WOUND	GAUZE PACK	TONGUE DEPRES.	TISSUE ADHES.	OTHER BAND.
5/17/96	3	96-21-10	A	300-2	N,TR	NE	NA	NA	NA	NA	NA	NA	NA
			B	300-2	N,TR,DF	NE	NA	NA	NA	NA	NA	NA	NA
			C	300-2	N,TR	SP	NA	NA	NA	NA	NA	NA	NA
			D	300-2	N,TR,DF	SP	NA	NA	NA	NA	NA	NA	NA
			E	300-2	N,TR	ESP	NA	NA	NA	NA	NA	NA	NA
			F	300-2	N,TR,DF	ESP	NA	NA	NA	NA	NA	NA	NA
5/20/96	6	96-21-10	A	300-2	N,TR	NE	NA	NA	NA	NA	NA	NA	NA
			B	300-2	N,TR,DF	NE	NA	NA	NA	NA	NA	NA	NA
			C	300-2	N,TR	SP	NA	NA	NA	NA	NA	NA	NA
			D	300-2	N,TR,DF	SP	NA	NA	NA	NA	NA	NA	NA
			E	300-2	N,TR	ESP	NA	NA	NA	NA	NA	NA	NA
			F	300-2	N,TR,DF	ESP	NA	NA	NA	NA	NA	NA	NA
5/24/96	10	96-21-10	A	300-2	N,TR	NE	NA	NA	NA	NA	NA	NA	NA
			B	300-2	N,TR,DF	NE	NA	NA	NA	NA	NA	NA	NA
			C	300-2	N,TR	SP	NA	NA	NA	NA	NA	NA	NA
			D	300-2	N,TR,DF	SP	NA	NA	NA	NA	NA	NA	NA
			E	300-2	N,TR	ESP	NA	NA	NA	NA	NA	NA	NA
			F	300-2	N,TR,DF	ESP	NA	NA	NA	NA	NA	NA	NA
5/28/96	14	96-21-10	A	300-2	N,TR	NE	NA	NA	NA	NA	NA	NA	NA
			B	300-2	N,TR,DF	NE	NA	NA	NA	NA	NA	NA	NA
			C	300-2	N,TR	SP	NA	NA	NA	NA	NA	NA	NA
			D	300-2	N,TR,DF	SP	NA	NA	NA	NA	NA	NA	NA
			E	300-2	N,TR	ESP	NA	NA	NA	NA	NA	NA	NA
			F	300-2	N,TR,DF	ESP	NA	NA	NA	NA	NA	NA	NA

DATE	STUDY DAY	ANIMAL ID	SITE ID VENTRAL	HD DOSE uL-hr	PRETX	ABRADE	TX	ANTIBIOT	GAUZE WOUND	GAUZE PACK	TONGUE DEPRES.	TISSUE ADHES.	OTHER BAND.
6/12/96	1	96-34-5	A	300-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			B	600-2	N,TR,TS	No	NA	NA	NA	NA	NA	NA	NA
			C	600-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			D	300-2	N,TR,TS	No	NA	NA	NA	NA	NA	NA	NA
			E	500-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			F	500-2	N,TR,TS	No	NA	NA	NA	NA	NA	NA	NA
6/13/96	2	96-34-5	A	300-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			B	600-2	N,TR,TS	No	NA	NA	NA	NA	NA	NA	NA
			C	600-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			D	300-2	N,TR,TS	No	NA	NA	NA	NA	NA	NA	NA
			E	500-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			F	500-2	N,TR,TS	No	NA	NA	NA	NA	NA	NA	NA
6/17/96	6	96-34-5	A	300-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			B	600-2	N,TR,TS	No	NA	NA	NA	NA	NA	NA	NA
			C	600-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			D	300-2	N,TR,TS	No	NA	NA	NA	NA	NA	NA	NA
			E	500-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			F	500-2	N,TR,TS	No	NA	NA	NA	NA	NA	NA	NA

DATE	STUDY DAY	ANIMAL ID	SITE ID VENTRAL	HD DOSE uL-hr	PRETX	ABRADE	TX	ANTIBIOT	GAUZE WOUND	GAUZE PACK	TONGUE DEPRES.	TISSUE ADHES.	OTHER BAND.
6/28/96	3	96-34-4	F	300-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			F1	300-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
7/1/96	6		D	300-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			D1	300-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
7/4/96	9		B	300-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			B1	300-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
7/7/96	12		A	300-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			A1	300-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
7/10/96	15		E	300-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			E1	300-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
7/13/96	18		C	300-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			C1	300-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA

DATE	STUDY DAY	ANIMAL ID	SITE ID VENTRAL	HD DOSE uL-hr	PRETX	ABRADE	TX	ANTIBIOT	GAUZE WOUND	GAUZE PACK	TONGUE DEPRES.	TISSUE ADHES.	OTHER BAND.
8/22/96	3	96-1-4	A	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			B	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			C	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			D	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			E	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			F	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
8/25/96	6	96-1-4	A	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			B	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			C	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			D	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			E	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			F	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
8/27/96	8	96-1-4	A	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			B	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			C	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			D	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			E	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			F	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA

DATE	STUDY DAY	ANIMAL ID	SITE ID VENTRAL	HD DOSE uL-hr	PRETX	ABRADE	TX	ANTIBIOT	GAUZE WOUND	GAUZE PACK	TONGUE DEPRES.	TISSUE ADHES.	OTHER BAND.
8/24/96	3	96-1-3	A	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			B	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			C	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			D	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			E	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			F	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
8/27/96	6	96-1-3	A	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			B	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			C	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			D	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			E	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			F	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
8/29/96	8	96-1-3	A	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			B	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			C	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			D	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			E	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			F	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA

DATE	STUDY DAY	ANIMAL ID	SITE ID VENTRAL	HD DOSE uL-hr	PRETX	ABRADE	TX	ANTIBIOT	GAUZE WOUND	GAUZE PACK	TONGUE DEPRES.	TISSUE ADHES.	OTHER BAND.
8/29/96	3	96-3-9	A	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			B	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			C	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			D	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			E	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			F	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
9/1/96	6	96-3-9	A	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			B	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			C	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			D	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			E	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			F	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
9/3/96	8	96-3-9	A	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			B	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			C	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			D	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			E	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			F	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA

DATE	STUDY DAY	ANIMAL ID	SITE ID VENTRAL	HD DOSE uL-hr	PRETX	ABRADE	TX	ANTIBIOT	GAUZE WOUND	GAUZE PACK	TONGUE DEPRES.	TISSUE ADHES.	OTHER BAND.
8/31/96	3	96-2-10	A	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			B	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			C	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			D	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			E	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			F	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
9/3/96	6	96-2-10	A	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			B	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			C	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			D	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			E	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			F	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
9/5/96	8	96-2-10	A	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			B	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			C	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			D	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			E	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			F	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA

DATE	STUDY DAY	ANIMAL ID	SITE ID VENTRAL	HD DOSE uL-hr	PRETX	ABRADE	TX	ANTIBIOT	GAUZE WOUND	GAUZE PACK	TONGUE DEPRES.	TISSUE ADHES.	OTHER BAND.
9/7/96	3	96-5-8	A	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			B	400-2	N	No	NA	NA	NA	NA	NA	NA	NA
			C	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			D	400-2	N	No	NA	NA	NA	NA	NA	NA	NA
			E	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			F	400-2	N	No	NA	NA	NA	NA	NA	NA	NA
9/10/96	6	96-5-8	A	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			B	400-2	N	No	NA	NA	NA	NA	NA	NA	NA
			C	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			D	400-2	N	No	NA	NA	NA	NA	NA	NA	NA
			E	400-2	N,TR	No	NA	NA	NA	NA	NA	NA	NA
			F	400-2	N	No	NA	NA	NA	NA	NA	NA	NA

DATE	STUDY DAY	ANIMAL ID	SITE ID VENTRAL	HD DOSE uL-hr	PRETX	ABRADE	TX	ANTIBIOT	GAUZE WOUND	GAUZE PACK	TONGUE DEPRES.	TISSUE ADHES.	OTHER BAND.
9/18/96	2	96-10-12	A	400-2	N	No	NA	NA	NA	NA	NA	NA	NA
			B	400-2	N	No	NA	NA	NA	NA	NA	NA	NA
			C	400-2	N	No	NA	NA	NA	NA	NA	NA	NA
			D	400-2	N	No	NA	NA	NA	NA	NA	NA	NA
			E	400-2	N	No	NA	NA	NA	NA	NA	NA	NA
			F	400-2	N	No	NA	NA	NA	NA	NA	NA	NA
9/19/96	2	96-10-11	A	400-2	N	No	NA	NA	NA	NA	NA	NA	NA
			B	400-2	N	No	NA	NA	NA	NA	NA	NA	NA
			C	400-2	N	No	NA	NA	NA	NA	NA	NA	NA
			D	400-2	N	No	NA	NA	NA	NA	NA	NA	NA
			E	400-2	N	No	NA	NA	NA	NA	NA	NA	NA
			F	400-2	N	No	NA	NA	NA	NA	NA	NA	NA
9/25/96	2	96-11-8	A	400-2	N	No	NA	NA	NA	NA	NA	NA	NA
			B	400-2	N	No	NA	NA	NA	NA	NA	NA	NA
			C	400-2	N	No	NA	NA	NA	NA	NA	NA	NA
			D	400-2	N	No	NA	NA	NA	NA	NA	NA	NA
			E	400-2	N	No	NA	NA	NA	NA	NA	NA	NA
			F	400-2	N	No	NA	NA	NA	NA	NA	NA	NA
9/26/96	2	96-11-11	A	400-2	N	No	NA	NA	NA	NA	NA	NA	NA
			B	400-2	N	No	NA	NA	NA	NA	NA	NA	NA
			C	400-2	N	No	NA	NA	NA	NA	NA	NA	NA
			D	400-2	N	No	NA	NA	NA	NA	NA	NA	NA
			E	400-2	N	No	NA	NA	NA	NA	NA	NA	NA
			F	400-2	N	No	NA	NA	NA	NA	NA	NA	NA

DATE	STUDY DAY	ANIMAL ID	SITE ID VENTRAL	HD DOSE uL-hr	PRETX	ABRADE	TX	ANTIBIOT	GAUZE WOUND	GAUZE PACK	TONGUE DEPRES.	TISSUE ADHES.	OTHER BAND.
10/17/96	2	96-18-8	A	400-2	N	No	SE	BACI	DR WAD	MG	NTD	NA	VW,S,AST
			B	400-2	N	No	SE	TH	DR FLD	MG	NTD	NA	VW,S,AST
			C	400-2	N	No	SE	BACI	DR WAD	MG	NTD	NA	VW,S,AST
			D	400-2	N	No	SE	TH	DR FLD	MG	NTD	NA	VW,S,AST
			E	400-2	N	No	SE	TH	DR WAD	MG	NTD	NA	VW,S,AST
			F	400-2	N	No	SE	TH	DR FLD	MG	NTD	NA	VW,S,AST
10/24/96	9	96-18-8	A	400-2	N	No	SE	TH	DR WAD	MG	NTD	TB	VW,S,AST
			B	400-2	N	No	SE	TH	DR FLD	MG	NTD	TB	VW,S,AST
			C	400-2	N	No	SE	TH	DR WAD	MG	NTD	TB	VW,S,AST
			D	400-2	N	No	SE	TH	DR FLD	MG	NTD	TB	VW,S,AST
			E	400-2	N	No	SE	TH	DR WAD	MG	NTD	TB	VW,S,AST
			F	400-2	N	No	SE	TH	DR FLD	MG	NTD	TB	VW,S,AST
10/31/96	16	96-18-8	A	400-2	N	No	SE	TH	VG,WD	MG	TD1	TB	VW,S,AST
			B	400-2	N	No	SE	TH	VG,WD	MG	TD1	TB	VW,S,AST
			C	400-2	N	No	SE	TH	VG,WD	MG	TD1	TB	VW,S,AST
			D	400-2	N	No	SE	TH	VG,WD	MG	TD1	TB	VW,S,AST
			E	400-2	N	No	SE	TH	VG,WD	MG	TD1	TB	VW,S,AST
			F	400-2	N	No	SE	TH	VG,WD	MG	TD1	TB	VW,S,AST
11/7/96	23	96-18-8	A	400-2	N	No	SE	NA	NA	NA	NA	NA	NA
			B	400-2	N	No	SE	NA	NA	NA	NA	NA	NA
			C	400-2	N	No	SE	NA	NA	NA	NA	NA	NA
			D	400-2	N	No	SE	NA	NA	NA	NA	NA	NA
			E	400-2	N	No	SE	NA	NA	NA	NA	NA	NA
			F	400-2	N	No	SE	NA	NA	NA	NA	NA	NA
11/14/96	30	96-18-8	A	400-2	N	No	SE	NA	NA	NA	NA	NA	NA
			B	400-2	N	No	SE	NA	NA	NA	NA	NA	NA
			C	400-2	N	No	SE	NA	NA	NA	NA	NA	NA
			D	400-2	N	No	SE	NA	NA	NA	NA	NA	NA
			E	400-2	N	No	SE	NA	NA	NA	NA	NA	NA
			F	400-2	N	No	SE	NA	NA	NA	NA	NA	NA
11/21/96	37	96-18-8	A	400-2	N	No	SE	NA	NA	NA	NA	NA	NA
			B	400-2	N	No	SE	NA	NA	NA	NA	NA	NA
			C	400-2	N	No	SE	NA	NA	NA	NA	NA	NA
			D	400-2	N	No	SE	NA	NA	NA	NA	NA	NA
			E	400-2	N	No	SE	NA	NA	NA	NA	NA	NA
			F	400-2	N	No	SE	NA	NA	NA	NA	NA	NA

APPENDIX B

DESCRIPTIONS OF TECHNIQUE USED TO PRODUCE PARTIAL- TO FULL-THICKNESS SKIN BURNS ON EACH ANIMAL

The pretreatment regime, dosing parameters, and results are presented for each animal, or group of animals in the order in which experiments were conducted. Animal 1 was pretreated on the dorsum with Nair®. Animals 2 through 16 were pretreated on the ventral abdomen with Nair®.

ANIMAL 1 (96-13-9)

Pretreatment and Dosing: Trypsin (Trypzyme-V®) was applied to the right side of the dorsum approximately 2 hr prior to dosing. The left side was tape stripped. The exposure time (1, 1.5 and 2 hr) was varied for the 200 μL HD volume applied to each site.
Results: Deep red circular lesions were observed on Day 6. Histopathologic examination of the biopsy revealed a necrotic epidermis with large areas of necrosis in the dermis, but no sloughing of damaged tissue nor deep wound.

ANIMAL 2 (96-13-7)

Pretreatment and Dosing: The Study Director in consultation with the COR decided to apply trypsin to the right side dose sites and papain to the left side dose sites of the ventral abdomen. Enzyme solutions were left on overnight. Also, alternate sites (opposite ventral midline) were abraded using either a 25 ga needle, sand paper, or emery paper. The HD volume applied to each site was increased to 500 μL and a 2 hr exposure was used.
Results: Day 5 biopsies indicated that deep full-thickness burns (to subcutaneous structures for sites A, B, C, and D and through dermal structures for sites E and F) were obtained.

ANIMALS 3 (96-21-7) and 4 (96-21-10)

Pretreatment and Dosing: Pretreatments and dosing parameters were the same for these two animals. Trypsin was applied overnight to the dose areas. Sites were abraded, the left side (sites B, D, and F) was swabbed with isopropyl alcohol before dosing, and an HD volume of 300 μL and a 2 hr exposure were used. Biopsy samples for histopathology were not collected from animal 3. The lesions on animal 3 were allowed to develop without any treatment. Clinical observation data were collected, and the appearance of the lesions remained unchanged for up to 20 days. Biopsies were taken from animal 4 on Days 3, 6, 10, and 14. Clinical evaluations were taken at similar time points in both animals. Difficulties with the grading system for both clinical observation and histopathology data became more apparent. Two grading systems were needed, one for wound development and one for wound healing. Descriptions of dose sites/lesions were made for both wound development and wound healing and modifications to the clinical observations data recordings were begun.
Results: Results indicated that, early in wound development, cells appeared normal or the epidermis necrotic. After approximately 6 days, the epidermis was completely necrotic with dermal infiltration of neutrophils (inflammation). Within 10 days the epidermis sloughed, and there were varying depths of dermal necrosis and inflammation of the deeper dermis. With more time, some neovascularization occurred, along with macrophage infiltration and fibroblast formation. Granulation tissue appeared to be extensive in the denuded wound areas and epithelialization occurred from the edges.

ANIMAL 5 (96-34-5)

Pretreatment and Dosing: The Study Director and COR decided that trypsin would be applied overnight on both sides, no abrading would be done, tape stripping would be done on one side only, and various HD volumes (300, 500, 600 μL) would be used in a 2 hr exposure.
Results: By Day 2 the epidermis was necrotic at all sites and one site showed epidermal-dermal separation in focal areas. The dermis did not appear necrotic. By Day 6, the epidermis was necrotic (full thickness, including basal layer) and separating from the dermis, but still covered the dermis. Dermal structures showed minimal to moderate inflammation depending upon dose volume. Tape stripping did not appear to enhance the severity of the lesions and thus was not used in the remaining animals.

ANIMAL 6 (96-34-4)

Pretreatment and Dosing: Animal 6 was treated as described for animal 5, except tape stripping was not used and a 300 μL HD dose volume was applied to all sites for 2 hr. The animal was evaluated histopathologically and clinically every three days for 18 days. Biopsies were taken from the most severe areas and peripherally from each site and from between sites.
Results: The epithelium died within 6-8 days, down to and including basal cells and basement membrane. Frequently the dermis was necrotic. Around Day 9 the necrotic epithelium sloughed, exposing the dermis, and the inflammation became more chronic (with macrophages, lymphocytes, and fibroblasts), developing into granulation tissue. The epithelium began regenerating about Day 12 from the sides of the wound and from surviving basal cells (possibly present in adnexal structures) in the less severe wounds. The new epithelium grew under a "protective" covering (scab). Less severe sites were covered with epithelium by Day 15, and some more severe sites were not covered by Day 18.

ANIMALS 7-10 (96-1-4, 96-1-3, 96-3-9, and 96-2-10, respectively)

Pretreatment and Dosing: Trypzyme-V® was applied to the dosing area overnight, except for Animal 10. For Animal 10, the USAMRICD Scientific Technical Objective (STO) representative, the COR, and the Study Director decided that trypsin would be applied overnight to the right side dosing sites only. As a result of miscommunications, the trypsin was applied to both sides, but was removed from the left side dosing sites after 1 hour. The HD dose volume used on these animals was increased to 400 μL per dose site and a 2 hr exposure was maintained.
Results: On Day 3, complete necrosis of the epidermis was observed across dosing sites, and included skin adnexal structures. Some sites showed minimal to marked neutrophil infiltration in the dermis and/or subcutis. Some sites showed minimal to moderate edema in the dermis and/or subcutis. The severity of edema and neutrophil infiltration varied from site to site. The most posterior sites appeared most severe with some arterioles showing necrotic muscular cells. By Day 6, there was full thickness necrosis of the epidermis with mild to marked neutrophil infiltration into the dermis and subcutis. Some sites appeared to have abscesses. In some sites, the dermis near the epidermis was necrotic. In the subcutaneous layer of some sites, mild to moderate edema was observed with some sites having marked neutrophil infiltration. Some sites had point ulcers in the epidermis or an intact epidermis that had areas

of separation. Day 8 biopsies indicated that full thickness necrosis of epidermis and dermis was present, with point or focal ulcers noted at some sites. In most sites there was some involvement of the subcutis. In some sites, bacterial colonies were observed in the dermis, particularly under the basal layer or where ulceration had occurred. There was no evidence of repair in any of the biopsies submitted as these were taken from the most severe area of each lesion. The general impression from these slides (including animal 11's Days 3 and 6) was that different sites produced the same lesion in the same animal at the same time point. As time progresses, the entire biopsy section becomes necrotic. The liquefaction necrosis observed was the type of lesion expected as a result of necrosis and degranulation of neutrophils.

ANIMAL 11 (96-5-8)

Pretreatment and Dosing: Pretreatments and treatments were as described for animals 7-10 above, except that trypsin was applied overnight to the right side. This regimen was to determine if removal of the keratin layer was needed.

Results: Day 3 results indicated that full thickness necrosis was observed across dosing sites with minimal to moderate infiltration of the dermis and subcutis, depending upon site. Foci of hemorrhage were observed at some sites. By Day 6, the entire biopsy section was necrotic, with moderate neutrophil and/or mixed inflammatory cell infiltration of the dermis and subcutis.

ANIMALS 12-15 (96-10-12, 96-10-11, 96-11-8, and 96-11-11, respectively)

Pretreatment and Dosing: Trypsin was not applied. The dose volume was 400 μL with a 2 hr exposure. The dose sites for these animals were evaluated on Day 2, prior to excision.

Results: In day 2 biopsies, full thickness necrosis of the epidermis and follicular epithelium were observed across the lesions on each animal. Minimal to mild infiltrate was observed in the dermis and subcutis, which were necrotic at some sites in some animals. There was edema in the subcutis routinely. Some lesions had foci of hemorrhage, primarily in the subcutis, but hemorrhage was not observed in each animal, nor in each lesion of an animal. A minute area of ulceration was observed in a single anterior-most wound. Animals 14 (only lesions A, D, and F were readable) and 15 (all sites readable) were also biopsied on Day 16. Dense granulation tissue with fibrosis and randomly distributed blood vessels was observed. Lesions with no epithelium generally showed necrotic neutrophils on the surface. Some lesions showed a thick layer of markedly hyperplastic epithelium with an irregular and atypical stratum malpighii, and numerous irregular dermal papillae. The superficial dermis was slightly edematous and neither hair follicles nor sweat glands were present. Some acute, necrotic inflammation was observed distributed throughout the sections reviewed.

ANIMAL 16 (96-18-8)

Pretreatment and Dosing: Nair® was applied to the ventral abdomen dosing area for approximately 7 min the day prior to dosing. No additional pretreatments were done. An HD volume of 400 μL was applied for 2 hr at each dosing site. Biopsies for each dosing site were collected and evaluated on Study Days 2, 9, 16, and 23.

Results: Day 2 biopsies showed that the epidermis was acutely necrotic, including most follicular epithelium and other adnexal structures. In general, most sites indicated full-thickness necrosis. Day 9 biopsies had a necrotic epithelium with small necrotic denuded areas and marked epithelial hyperplasia present in the area adjacent to the denuded area (chronic regeneration to cover the wound). Epithelialization was observed under the necrotic area. Moderate to marked granulation tissue (bands of fibrous tissue with chronic inflammation), depending upon the lesion evaluated, was observed in the subcutaneous tissue and extended under the denuded area. Day 16 biopsies showed some wounds denuded, while others indicated mild to moderate healing in the section presented. Epithelium was moderately hyperplastic and either completely covered the section provided or was in the area adjacent to the denuded area. Mild to marked granulation tissue completely filled the dermis and subcutis. The general impression was that healing was mild to moderate depending upon the section evaluated. Day 23 biopsies indicated that the lesions were full thickness burns. Each lesion was completely ulcerated, except for site D, in which approximately three-quarters of the area sectioned was ulcerated. Granulation was moderate to marked and epithelialization (hyperplasia) was mild to moderate. The lesions, rated for their completeness of healing, ranged from mild to moderate. Lesions E and F were excised and submitted for evaluation since these areas ulcerated after they had appeared to be healed. Both sections contained a central area of acute hemorrhage with moderate to severe suppurative inflammation. Epithelium at the edges of the central hemorrhagic area was moderately hyperplastic. The underlying dermis was fibrotic and appeared to be the end-stage of a significant degree of granulation. The superficial central area of these sections contained residual bacteria and/or inflammation, which resulted in incomplete adhesion of the encroaching overlying epidermis, even though grossly the epidermis appeared to cover the lesion.

APPENDIX C

CHEMISTRY REPORT

Battelle
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Project Number G1555-33ACH

Internal Distribution

Tim Hayes
File copy

Date October 8, 1997

To Frances Reid

From Janet G. Ricks

Subject Dose Confirmation - Task 33

Attached is a table summarizing dosing dates, laboratory sample numbers, expected and measured dose concentrations and percent of expected for Task 33 from April 10 to October 15, 1996.

All samples were analyzed in triplicate by gas chromatography using a flame ionization detector. The percent of expected, in all cases, is well within the range of ± 20%.

DOSE CONFIRMATION ANALYSIS FOR TASK 33

Dosing Date	Sample Number	Expected Dose Concentration (mg/ml)	Measured Dose Concentration (mg/ml)	Percent of Expected
4-10-96	50015-12-01	0.930	0.922	99.2
4-24-96	50015-12-02	1.16	1.14	98.4
5-6-96	50015-12-03	1.39	1.36	97.8
5-14-96	50015-12-04	1.39	1.36	97.6
6-11-96	50015-12-05	1.39	1.38	99.5
6-11-96	50015-12-06	1.16	1.14	98.4
6-11-96	50015-12-07	1.39	1.37	98.6
6-25-96	50015-12-08	1.39	1.35	97.2
8-19-96	50015-12-09	0.930	0.903	97.1
8-21-96	50015-12-10	0.930	0.912	98.1
8-26-96	50015-14-01	0.930	0.897	96.5
8-28-96	50015-14-02	0.930	0.915	98.4
9-4-96	50015-14-03	0.930	0.914	98.3
9-16-96	50015-14-04	0.755	0.693	91.8
9-17-96	50015-14-05	0.930	0.923	99.3
9-23-96	50015-14-06	0.930	0.906	97.4
9-24-96	50015-14-07	0.930	0.907	97.5
10-15-96	50015-14-08	0.930	0.909	97.7

APPENDIX D

STATISTICS REPORT

Project Number G1555-B33ASTAT (3104)

Battelle
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Internal Distribution

Department Files
R. Menton
N. Niemuth
J. Nagaraja
RMO

Date September 30, 1997

To Frances Reid

From Nancy Niemuth

Subject **Task 94-33**

s:\niem\mref\task33\Reid Memo.wpd
(s:\.....Phase I Report.wpd)

Attached is a statistical report on clinical observations and histopathologic data collected in MREF Task 94-33, Phase I (First 16 Animals). The statistical summary section was written so that it may be incorporated into your draft report, between the Experimental Design and Conclusions sections. A WordPerfect 8 file with the text, tables, and figures will be sent via cc:Mail for this purpose.

NN:kc
Attachment

For Review and Approval

	Name	Initials	Date
Originator	N. Niemuth	N	10/1/97
Concurrence	R. Menton	R bm	10/3/97
Approved	J. Orban	JEC	10/5/97

STATISTICAL REPORT FOR MREF TASK 94-33, PHASE I (FIRST 16 ANIMALS)

BACKGROUND

This report summarizes data from clinical observation and histopathological examination of HD-induced dermal lesions on the first 16 animals in Phase 1 of Task 94-33. Six sites on each animal were pretreated and dosed as described in Table 1. A variety of pretreatment and HD-dose combinations were tested to determine a regimen that would consistently produce deep partial or full-thickness burns.

STATISTICAL SUMMARY

Due to the exploratory nature of these experiments, the statistical analysis focused on summarizing the results for the pretreatment and HD-dose combination that was selected for use in future phases of this task. The last five animals (96-10-12, 96-10-11, 96-11-8, 96-11-11, and 96-18-8) were pretreated with Nair only and dosed with 400 μL for 2 hours on all six sites. In addition, one animal (96-5-8) received this combination on the left side (sites B, D, and F). Table 2 summarizes the study days on which wounds were evaluated on these animals.

HISTOPATHOLOGY

The incidence of histopathology endpoints for animals pretreated with Nair only and dosed with 400 μL HD for 2 hours is summarized in Table 3. Two conditions, microblisters and infection, were not present in these animals. In addition, dermal hemorrhage was observed infrequently. The following conditions were observed in at least half the sites (n=30) on study day 2: epidermal necrosis (93.3%), follicular necrosis (60.0), dermal neutrophil infiltration (73.3), subcutaneous hemorrhage (50.0), subcutaneous edema (76.7) and subcutaneous neutrophil infiltration (73.3). Although fewer sites were examined on later study days, high incidences of dermal neutrophil infiltration and subcutaneous neutrophil infiltration were observed through day 23; epidermal necrosis and follicular necrosis continued to be evident through study day 6, but not later; and subcutaneous hemorrhage and subcutaneous edema were not observed after study day 3, with the exception that subcutaneous hemorrhage was observed in two sites on day 23. As indicated above, these two sites tore open after they appeared to have healed. Two conditions were observed with high incidence on the middle study days: dermal necrosis on days 3-9 and subcutaneous necrosis on days 3-16. Incidences of dermal edema and neovascularization peaked on study day 9, each present in 3 of the 6 sites examined on that day. The remaining histopathological indicators were most often present on later study days. Granulation, re-epithelialization, and slough were observed in most sites on days 9-23; epidermal ulceration and epidermal neutrophil infiltration were observed on days 9 and 23, but not on day 16. Epidermal neutrophil infiltration was also observed in 2 of 3 sites on day 6.

A more complete summary of histopathologic evaluations is presented in Tables A-1 through A-4 in Addendum A. These tables present incidence of histopathologic endpoints on study day two and later, for all pretreatment and HD-dose combinations where two or more sites were treated.

CLINICAL OBSERVATIONS

Descriptive statistics for clinical observations are presented in Table 4 for animals pretreated with Nair only and dosed with 400 μL HD for 2 hours. Exudate, erytherma, edema, necrosis, and eschar were evaluated on days 2, 3, and 6. Eschar was not present on these days and was excluded from the table. A clinical wound severity score (WSS) was calculated as the sum of these scores. To evaluate wound healing, observations of exudate, inflammation, granulation, contraction, infection, vascularization, and epithelialization were taken on study days 9, 16, 23, 30, and 37 for animal 96-18-8. Wound size (WS) was measured for each site, on each study day.

Erythema, necrosis, and edema were present in all sites on study day 2, except that necrosis was not present in 1 site on animal 96-10-11. Erythema scores tended to be most severe, comprising approximately one-half of WSS. Exudate was observed infrequently, except on animal 96-18-8, where it was observed in 4 of 6 sites. When observed, exudate was mild. Figure 1 presents the mean WSS for each animal, overlaid on the observed values for each site. From the figure, it is apparent that the WSS varies considerably both within sites on an animal and between animals.

Wound size data are presented in Figures 2 and 3. Figure 2 illustrates the mean WS for each animal on study day 2, overlaid on the observed values for each site. Figure 3 displays the mean WS plotted against time for animal 96-18-8, overlaid on the observed values for each site. On study day 2, WS appears to be fairly consistent within sites on an animal and between animals. For animal 96-18-8, WS is greatest on study day 9 and variability appears to increase with time. However, WS measurements on day 30 appear to be inconsistent with measurements on days 23 and 37. This is attributed to differences among individuals taking the measurements, rather than rapid changes in the wounds during this period.

Figure 4 presents the mean score for each wound healing parameter, averaged over the 6 sites on animal 96-18-8, plotted against time. The wound healing scores tended to decrease over time, with the exception that inflammation increased through study day 23 and was, in general, not observed thereafter. In addition, granulation scores were higher on day 30 compared to earlier examinations. Granulation scores were not made on study day 37, as the wounds appeared to be completely healed.

Analysis of variance (ANOVA) models were fitted to the WSS and WS data collected on study day 2, to assess the animal-to-animal variability and to determine whether there were significant differences among sites. Appropriate contrasts were used to assess whether there were differences between anterior and posterior sites. The ANOVA model took the following form:

$$Y_{ij}=\mu+\alpha_i+\gamma_j+\epsilon_{ij}$$

where Y_{ij} is the observed WSS or WS for site i on animal j, μ is the average WSS or WS, α_i is a fixed site effect, γ_j is a random animal effect, and ϵ_{ij} is a random error term. The SAS (ver. 6.12) GLM procedure was used to fit the ANOVA models.

No significant effects were detected in the analysis of WS. There was significant animal-to-animal variability in WSS ($p<0.001$, $\sigma_{animal}=1.6$). Although the overall site effect was not statistically significant ($p=0.217$), WSS's were significantly greater on posterior sites than anterior sites ($p=0.025$, estimated difference in means [±SE] = 3.1 [±1.3]). These results are not contradictory. It is sometimes the case that noise in the data may cloud an effect in the overall test, but that a specific effect of interest may be detected by an appropriate hypothesis test.

Table 1. Pretreatment and Dosing Summary for First Sixteen Animals Used to Develop Sulfur Mustard-Induced Chemical Burns.

Animal Number	Animal ID	HD Dose[1] (μL-hr)	Right Side Pretreatments									Left Side Pretreatments								
			Site	Nair[2]	Trypsin[3]	Papain[4]	Tape Strip	Sand paper	Emery Paper	Needle	Defat	Site	Nair[2]	Trypsin[3]	Papain[4]	Tape Strip	Sand paper	Emory Paper	Needle	Defat
1	96-13-9[5]	200-2 (A&B)	B	x	x							A	x			x				
		200-1.5(C&D)	D	x	x							C	x			x				
		200-1 (E&F)	F	x	x							E	x			x				
2	96-13-7	500-2	A	x	x					x		B	x		x				x	
			C	x	x			x				D	x		x		x			
			E	x	x							F	x		x					
3 & 4	96-21-7 96-21-10	300-2	A	x	x					x		B	x	x					x	x
			C	x	x			x				D	x	x			x			x
			E	x	x				x			F	x	x				x		x
5	96-34-5	300-2 (A&D)	A	x	x							B	x	x		x				
		500-2 (B&C)	C	x	x							D	x	x		x				
		600-2 (E&F)	E	x	x							F	x	x		x				
6	96-34-4	300-2	A	x	x							B	x	x						
			C	x	x							D	x	x						
			E	x	x							F	x	x						
7-10	96-1-4 96-1-3 96-3-9 96-2-10	400-2	A	x	x							B	x	x						
			C	x	x							D	x	x						
			E	x	x							F	x	x						

Table 1. Pretreatment and Dosing Summary for First Sixteen Animals Used to Develop Sulfur Mustard-Induced Chemical Burns (continued).

Animal Number	Animal ID	HD Dose[1] (μL-hr)	Right Side Pretreatments									Left Side Pretreatments								
			Site	Nair[2]	Trypsin[3]	Papain[4]	Tape Strip	Sand paper	Emery Paper	Needle	Defat	Site	Nair[2]	Trypsin[3]	Papain[4]	Tape Strip	Sand paper	Emory Paper	Needle	Defat
11	96-5-8	400-2	A	x	x							B	x							
			C	x	x							D	x							
			E	x	x							F	x							
12-15	96-10-12 96-10-11 96-11-8 96-11-11	400-2	A	x								B	x							
			C	x								D	x							
			E	x								F	x							
16	96-18-8	400-2	A	x								B	x							
			C	x								D	x							
			E	x								F	x							

[1] All HD doses were applied for 2 hours, except for animal 96-13-9. On this animal, sites A & B were dosed for 2 hrs, C & D were dosed for 1.5 hours, and E & F were dosed for 1 hour.

[2] Nair was generally applied for 7 minutes. Occasionally, two applications were necessary for complete hair removal.

[3] Trypsin was applied overnight, except for animal 96-13-9. Trypsin was applied for 2 hours on this animal.

[4] Papain was applied overnight.

[5] Animal 96-13-19 was dosed on the dorsum. All other animals were dosed on the ventral abdomen.

Table 2. Summary of Study Days on Which Histopathological Evaluations and Clinical Observations Were Made for Animals Pretreated with Nair Only and Dosed with 400 μL HD for 2 Hours.

	Histopathologic Evaluation (Study Day)						Clinical Observations (Study Day)							
Animal ID	2	3	6	9	16	23	2	3	6	9	16	23	30	37
96-5-8*		x	x					x	x					
96-10-11	x						x							
96-10-12	x						x							
96-11-8	x				x		x				x**			
96-11-11	x				x		x							
96-18-8	x			x	x	x	x			x	x	x	x	x

* Three sites on this animal received the Nair only pretreatment.

** Clinical observations were collected on day 16, however, the endpoints examined were not consistent with those collected for animal 96-18-8 on that day.

Table 3. Incidence of Histopathology Endpoints on Sites Pretreated with Nair only and Dosed with 400 μL for Two Hours.

Histopathology Endpoint	Incidence [Number Observed / Number Examined (%)]					
	Nair Only Pretreatment					
	Day 2	Day 3	Day 6	Day 9	Day 16	Day 23
Epidermal Necrosis	28/30(93.3)	3/3(100.0)	3/3(100.0)	1/3(33.3)	0/12(0.0)	N/A
Follicular Necrosis	18/30(60.0)	3/3(100.0)	3/3(100.0)	1/3(33.3)	0/12(0.0)	N/A
Epidermal Ulceration	1/30(3.3)	0/3(0.0)	0/3(0.0)	4/6(66.7)	2/13(15.4)	6/6(100.0)
Epidermal Neutrophil Infiltration	1/30(3.3)	0/3(0.0)	2/3(66.7)	4/6(66.7)	0/13(0.0)	5/6(83.3)
Microblister	0/30(0.0)	0/3(0.0)	0/3(0.0)	0/6(0.0)	0/13(0.0)	0/6(0.0)
Slough	1/30(3.3)	0/3(0.0)	0/3(0.0)	5/6(83.3)	8/13(61.5)	6/6(100.0)
Dermal Hemorrhage	0/30(0.0)	1/3(33.3)	0/3(0.0)	2/6(33.3)	0/13(0.0)	2/6(33.3)
Dermal Necrosis	9/30(30.0)	3/3(100.0)	3/3(100.0)	3/6(50.0)	1/13(7.7)	0/6(0.0)
Dermal Ulceration	0/30(0.0)	0/3(0.0)	0/3(0.0)	1/6(16.7)	0/13(0.0)	6/6(100.0)
Dermal Neutrophil Infiltration	22/30(73.3)	3/3(100.0)	3/3(100.0)	6/6(100.0)	12/13(92.3)	5/6(83.3)
Dermal Edema	5/30(16.7)	0/3(0.0)	0/3(0.0)	3/6(50.0)	1/13(7.7)	0/6(0.0)
Neovascularization	0/30(0.0)	0/3(0.0)	0/3(0.0)	3/6(50.0)	2/13(15.4)	0/6(0.0)
Granulation	0/30(0.0)	0/3(0.0)	1/3(33.3)	6/6(100.0)	13/13(100.0)	6/6(100.0)
Subcutaneous Hemorrhage	15/30(50.0)	1/3(33.3)	0/3(0.0)	0/6(0.0)	0/13(0.0)	2/6(33.3)
Subcutaneous Edema	23/30(76.7)	1/3(33.3)	0/3(0.0)	0/6(0.0)	0/13(0.0)	0/6(0.0)
Subcutaneous Neutrophil Infiltration	22/30(73.3)	2/3(66.7)	3/3(100.0)	6/6(100.0)	12/13(92.3)	5/6(83.3)
Subcutaneous Necrosis	2/30(6.7)	3/3(100.0)	3/3(100.0)	3/6(50.0)	7/13(53.8)	0/6(0.0)
Re-epithelialization	0/30(0.0)	0/3(0.0)	0/3(0.0)	5/6(83.3)	7/13(53.8)	6/6(100.0)
Infection	0/30(0.0)	0/3(0.0)	0/3(0.0)	0/6(0.0)	0/13(0.0)	0/6(0.0)

Table 4. Descriptive Statistics of Clinical Observations Parameters on Sites Pretreated with Nair and Dosed with 400 μL for Two Hours, by Study Day.

Study Day	Clinical Observation Parameters	N	Mean	Std. Deviation	Minimum	Maximum
2	Exudate	30	0.18	0.41	0.00	1.50
	Erythema	30	4.78	0.41	3.25	5.50
	Edema	30	1.94	0.72	0.75	3.00
	Necrosis	30	1.79	1.33	0.00	4.00
	WSS	30	9.27	2.11	5.00	12.00
	WS (mm^2)	30	1406.07	113.59	1114.48	1621.06
3	Exudate	3	1.00	0.87	0.50	2.00
	Erythema	3	5.33	0.14	5.25	5.50
	Edema	3	1.67	0.58	1.00	2.00
	Necrosis	3	1.25	0.25	1.00	1.50
	WSS	3	10.23	0.76	9.65	11.08
	WS (mm^2)	3	1358.74	70.07	1317.11	1439.63
6	Exudate	3	0.00	0.00	0.00	0.00
	Erythema	3	5.00	0.00	5.00	5.00
	Edema	3	1.25	0.66	0.75	2.00
	Necrosis	3	4.00	0.00	4.00	4.00
	WSS	3	11.25	0.66	10.75	12.00
	WS (mm^2)	3	1613.99	40.81	1590.43	1661.12
9	Exudate	6	1.00	0.00	1.00	1.00
	Inflammation	6	1.54	0.81	1.00	3.00
	Granulation	6	1.83	0.70	1.00	3.00
	Contraction	6	2.00	0.00	2.00	2.00
	Infection	6	1.25	0.22	1.00	1.50
	Vascularization	6	1.67	1.21	0.00	3.00
	Epithelization	6	2.17	0.89	1.00	3.00
	WS (mm^2)	4	1870.82	246.92	1624.20	2120.58

Table 4. Descriptive Statistics of Clinical Observation Parameters on Sites Pretreated with Nair and Dosed with 400 μL for Two Hours, by Study Day (continued).

Study Day	Clinical Observation Parameters	N	Mean	Std. Deviation	Minimum	Maximum
16	Exudate	6	0.29	0.46	0.00	1.00
	Inflammation	6	1.79	1.42	0.00	3.50
	Granulation	6	1.00	0.00	1.00	1.00
	Contraction	6	1.00	0.00	1.00	1.00
	Infection	6	0.17	0.41	0.00	1.00
	Vascularization	6	0.58	1.43	0.00	3.50
	Epithelization	6	0.00	0.00	0.00	0.00
	WS (mm^2)	6	894.05	235.58	640.88	1328.89
23	Exudate	6	0.00	0.00	0.00	0.00
	Inflammation	6	1.96	1.10	0.00	3.00
	Granulation	6	1.00	0.00	1.00	1.00
	Contraction	6	1.00	0.00	1.00	1.00
	Infection	6	0.33	0.52	0.00	1.00
	Vascularization	6	0.00	0.00	0.00	0.00
	Epithelization	6	0.00	0.00	0.00	0.00
	WS (mm^2)	6	653.45	433.84	319.66	1476.55
30	Exudate	6	0.13	0.21	0.00	0.50
	Inflammation	6	0.00	0.00	0.00	0.00
	Granulation	6	3.00	0.00	3.00	3.00
	Contraction	6	0.67	1.03	0.00	2.00
	Infection	6	0.00	0.00	0.00	0.00
	Vascularization	6	0.00	0.00	0.00	0.00
	Epithelization	6	0.29	0.46	0.00	1.00
	WS (mm^2)	3	115.72	24.85	94.25	142.94
37	Exudate	6	0.00	0.00	0.00	0.00
	Inflammation	6	0.17	0.41	0.00	1.00
	Granulation *	0	.	.	.	.
	Contraction *	2	0.63	0.53	0.25	1.00
	Infection	6	0.00	0.00	0.00	0.00
	Vascularization *	2	0.50	0.00	0.50	0.50
	Epithelization	6	0.29	0.51	0.00	1.25
	WS (mm^2)	6	639.05	495.63	169.65	1418.43

* Condition could not be observed, as wounds appeared to be completely healed.

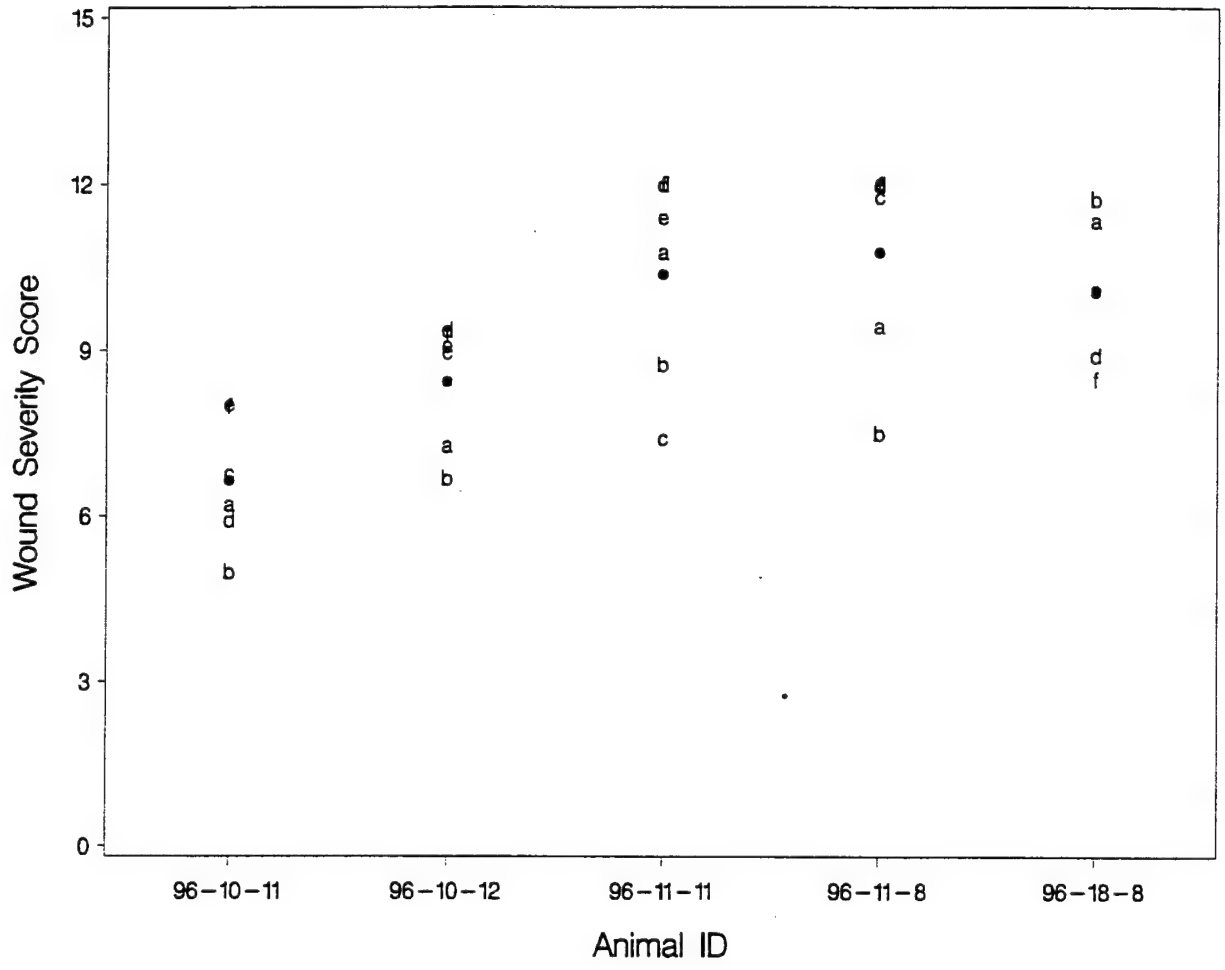

Figure 1. Wound Severity Score (WSS) of Five Different Animals on Study Day 2. Mean WSS (•) Overlaid on Observed Values for Sites A-F.

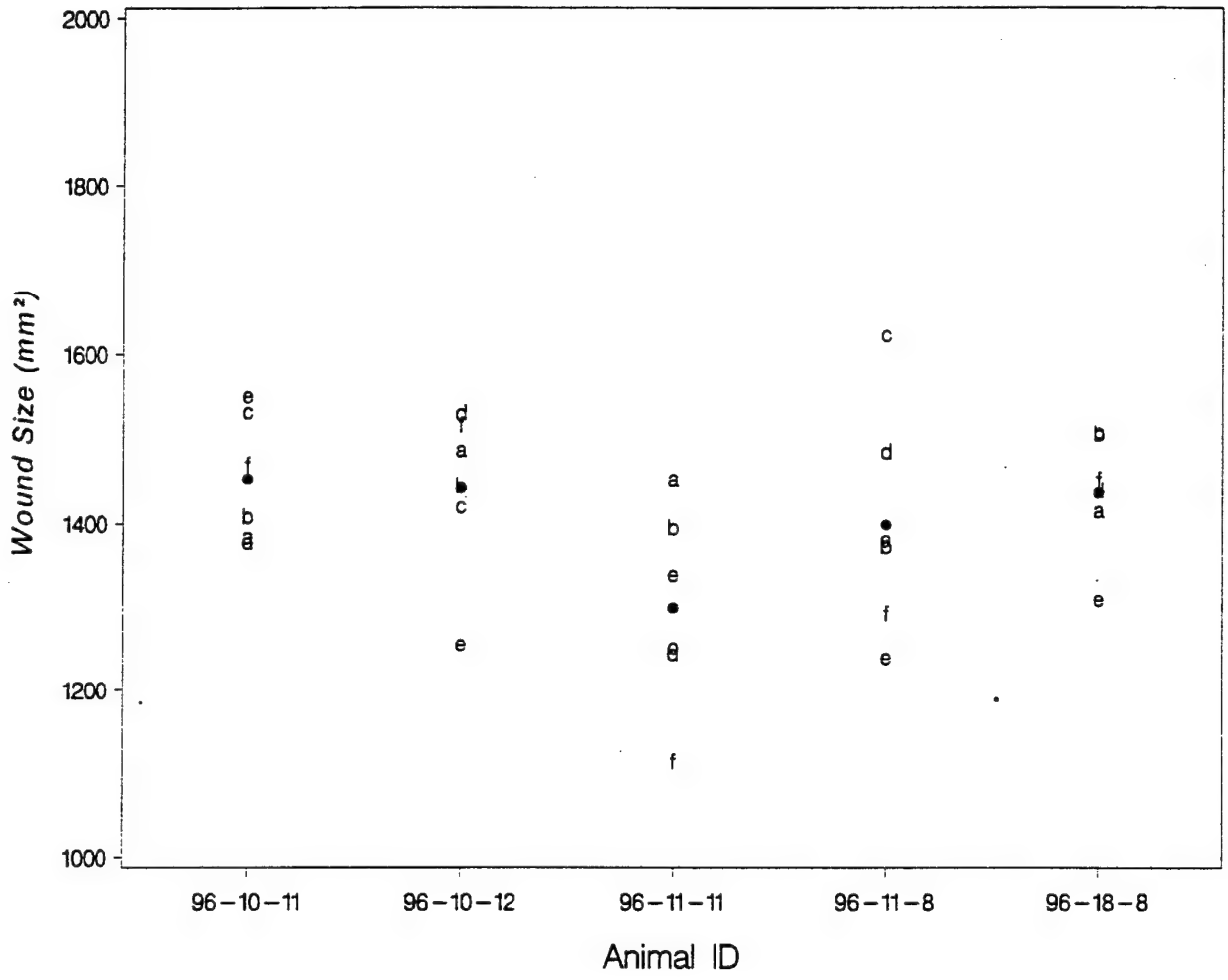

Figure 2. Wound Size (WS) of Five Different Animals on Study Day 2. Mean WS (•) Overlaid on Observed Values for Sites A-F.

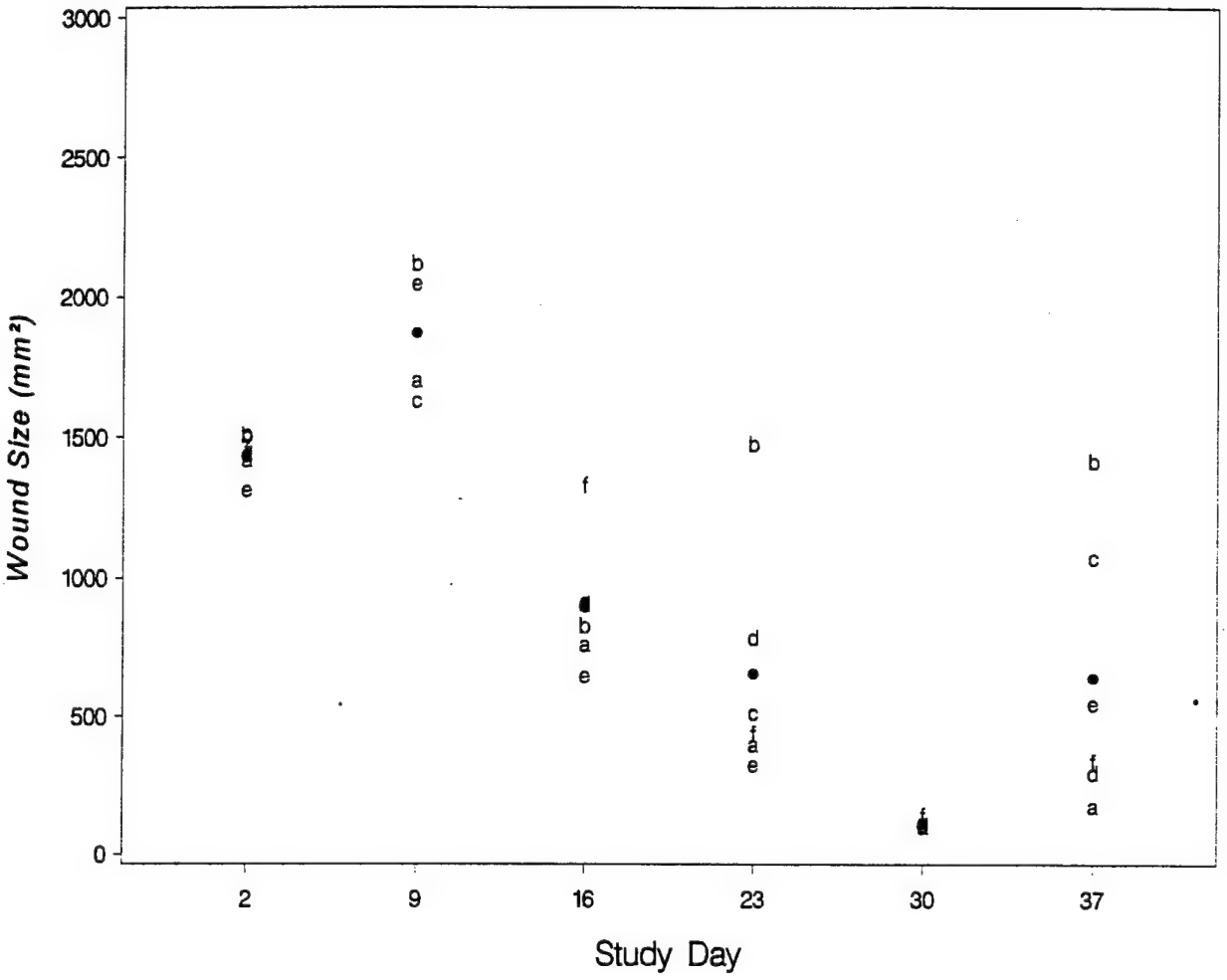

Figure 3. Wound Size (WS) for Animal 96-18-8 on Study Days 2, 9, 16, 23, 30, and 37. Mean WS (•) Overlaid on Observed Values for Sites A-F.

WS was not measured in sites D and F on study day 9, nor in sites B, C, and E on study day 30.

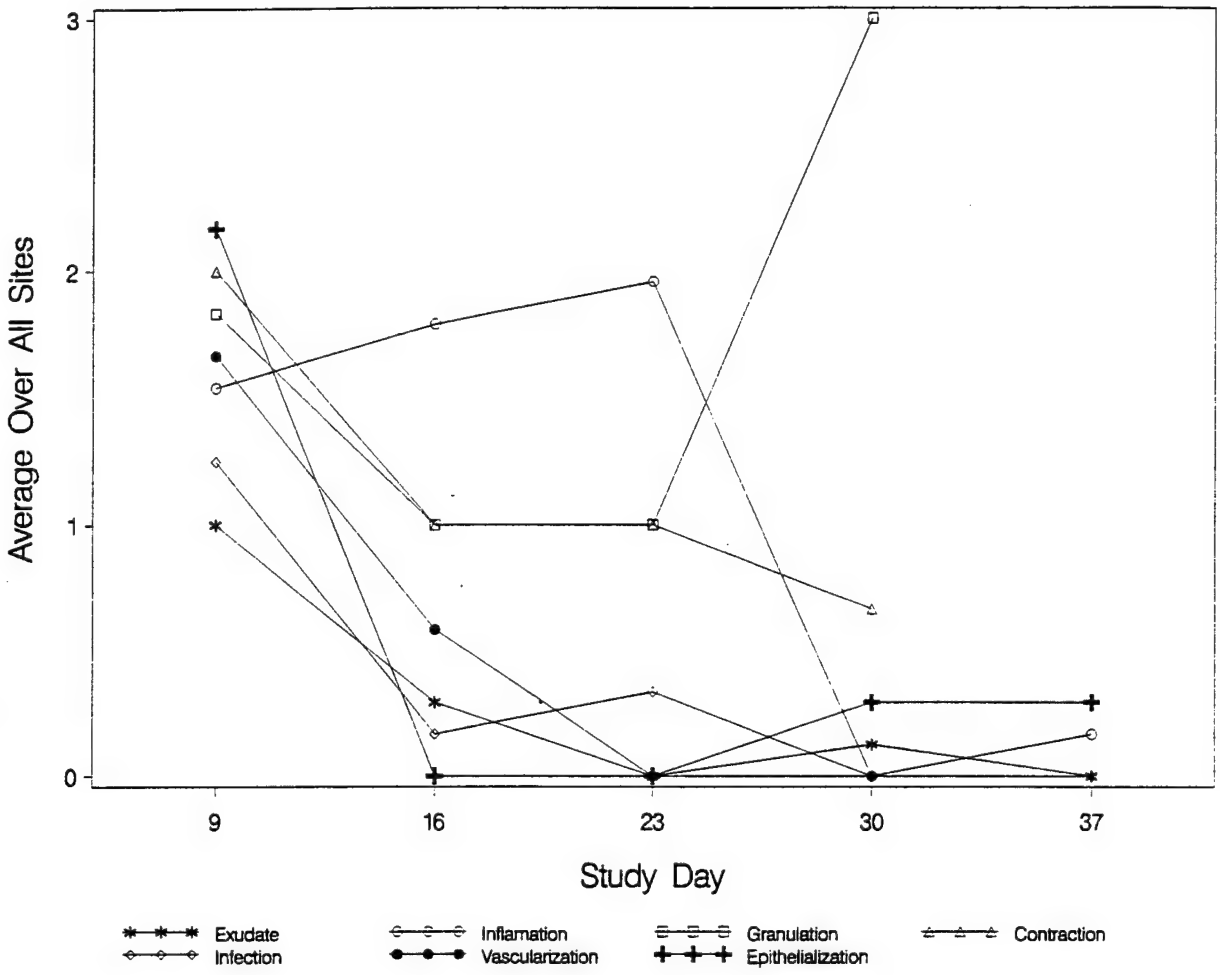

Figure 4. Comparison of Clinical Observation Parameters Averaged over All Sites on Study Days 9, 16, 23, 30, and 37, for Animal 96-18-8.

ADDENDUM A

SUMMARY OF HISTOPATHOLOGY EVALUATIONS

Table A-1. Incidence of Histopathology Endpoints on Sites Dosed with 300 μL HD for Two Hours.

Histopathology Endpoint	Incidence [Number Observed / Number Examined (%)]						
	Nair and Trypsin Pretreatment						
	Day 2	Day 3	Day 6	Day 9	Day 12	Day 15	Day 18
Epidermal Necrosis	2/2(100.0)	2/2(100.0)	4/4(100.0)	2/2(100.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)
Follicular Necrosis	0/2(0.0)	1/2(50.0)	0/4(0.0)	1/2(50.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)
Epidermal Ulceration	0/2(0.0)	0/2(0.0)	0/4(0.0)	2/2(100.0)	2/2(100.0)	1/2(50.0)	0/2(0.0)
Epidermal Neutrophil Infiltration	0/2(0.0)	1/2(50.0)	2/4(50.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)
Microblister	1/2(50.0)	0/2(0.0)	3/4(75.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)
Slough	0/2(0.0)	0/2(0.0)	0/4(0.0)	1/2(50.0)	2/2(100.0)	1/2(50.0)	0/2(0.0)
Dermal Hemorrhage	0/2(0.0)	1/2(50.0)	3/4(75.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)
Dermal Necrosis	0/2(0.0)	1/2(50.0)	0/4(0.0)	2/2(100.0)	2/2(100.0)	2/2(100.0)	0/2(0.0)
Dermal Ulceration	0/2(0.0)	0/2(0.0)	0/4(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)
Dermal Neutrophil Infiltration	0/2(0.0)	2/2(100.0)	3/4(75.0)	2/2(100.0)	2/2(100.0)	2/2(100.0)	1/2(50.0)
Dermal Edema	0/2(0.0)	0/2(0.0)	0/4(0.0)	1/2(50.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)
Neovascularization	0/2(0.0)	0/2(0.0)	0/4(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)	1/2(50.0)
Granulation	0/2(0.0)	0/2(0.0)	0/4(0.0)	0/2(0.0)	2/2(100.0)	1/2(50.0)	2/2(100.0)
Subcutaneous Hemorrhage	0/2(0.0)	0/2(0.0)	0/4(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)
Subcutaneous Edema	0/2(0.0)	0/2(0.0)	0/4(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)
Subcutaneous Neutrophil Infiltration	0/2(0.0)	0/2(0.0)	0/4(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)
Subcutaneous Necrosis	0/2(0.0)	0/2(0.0)	0/4(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)
Re-epithelialization	0/2(0.0)	0/2(0.0)	1/4(25.0)	0/2(0.0)	1/2(50.0)	1/2(50.0)	2/2(100.0)
Infection	0/2(0.0)	0/2(0.0)	1/4(25.0)	1/2(50.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)

Table A-2. Incidence of Histopathology Endpoints on Sites Pretreated with Nair Only and Dosed with 400 μL HD for Two Hours.

Histopathology Endpoint	Incidence [Number Observed / Number Examined (%)]					
	Nair Only Pretreatment					
	Day 2	Day 3	Day 6	Day 9	Day 16	Day 23
Epidermal Necrosis	28/30(93.3)	3/3(100.0)	3/3(100.0)	1/3(33.3)	0/12(0.0)	N/A
Follicular Necrosis	18/30(60.0)	3/3(100.0)	3/3(100.0)	1/3(33.3)	0/12(0.0)	N/A
Epidermal Ulceration	1/30(3.3)	0/3(0.0)	0/3(0.0)	4/6(66.7)	2/13(15.4)	6/6(100.0)
Epidermal Neutrophil Infiltration	1/30(3.3)	0/3(0.0)	2/3(66.7)	4/6(66.7)	0/13(0.0)	5/6(83.3)
Microblister	0/30(0.0)	0/3(0.0)	0/3(0.0)	0/6(0.0)	0/13(0.0)	0/6(0.0)
Slough	1/30(3.3)	0/3(0.0)	0/3(0.0)	5/6(83.3)	8/13(61.5)	6/6(100.0)
Dermal Hemorrhage	0/30(0.0)	1/3(33.3)	0/3(0.0)	2/6(33.3)	0/13(0.0)	2/6(33.3)
Dermal Necrosis	9/30(30.0)	3/3(100.0)	3/3(100.0)	3/6(50.0)	1/13(7.7)	0/6(0.0)
Dermal Ulceration	0/30(0.0)	0/3(0.0)	0/3(0.0)	1/6(16.7)	0/13(0.0)	6/6(100.0)
Dermal Neutrophil Infiltration	22/30(73.3)	3/3(100.0)	3/3(100.0)	6/6(100.0)	12/13(92.3)	5/6(83.3)
Dermal Edema	5/30(16.7)	0/3(0.0)	0/3(0.0)	3/6(50.0)	1/13(7.7)	0/6(0.0)
Neovascularization	0/30(0.0)	0/3(0.0)	0/3(0.0)	3/6(50.0)	2/13(15.4)	0/6(0.0)
Granulation	0/30(0.0)	0/3(0.0)	1/3(33.3)	6/6(100.0)	13/13(100.0)	6/6(100.0)
Subcutaneous Hemorrhage	15/30(50.0)	1/3(33.3)	0/3(0.0)	0/6(0.0)	0/13(0.0)	2/6(33.3)
Subcutaneous Edema	23/30(76.7)	1/3(33.3)	0/3(0.0)	0/6(0.0)	0/13(0.0)	0/6(0.0)
Subcutaneous Neutrophil Infiltration	22/30(73.3)	2/3(66.7)	3/3(100.0)	6/6(100.0)	12/13(92.3)	5/6(83.3)
Subcutaneous Necrosis	2/30(6.7)	3/3(100.0)	3/3(100.0)	3/6(50.0)	7/13(53.8)	0/6(0.0)
Re-epithelialization	0/30(0.0)	0/3(0.0)	0/3(0.0)	5/6(83.3)	7/13(53.8)	6/6(100.0)
Infection	0/30(0.0)	0/3(0.0)	0/3(0.0)	0/6(0.0)	0/13(0.0)	0/6(0.0)

Table A-3. Incidence of Histopathology Endpoints on Sites Pretreated with Nair and Trypsin and Dosed with 400 μL HD for Two Hours.

Histopathology Endpoint	Incidence [Number Observed / Number Examined (%)]					
	Nair and Trypsin Pretreatment			Nair and Trypsin (1-hr) Pretreatment [1]		
	Day 3	Day 6	Day 8	Day 3	Day 6	Day 8
Epidermal Necrosis	24/24(100.0)	24/24(100.0)	21/21(100.0)	3/3(100.0)	3/3(100.0)	3/3(100.0)
Follicular Necrosis	9/24(37.5)	11/24(45.8)	9/21(42.9)	1/3(33.3)	2/3(66.7)	3/3(100.0)
Epidermal Ulceration	0/24(0.0)	4/24(16.7)	7/21(33.3)	0/3(0.0)	1/3(33.3)	2/3(66.7)
Epidermal Neutrophil Infiltration	3/24(12.5)	10/24(41.7)	9/21(42.9)	0/3(0.0)	2/3(66.7)	2/3(66.7)
Microblister	0/24(0.0)	0/24(0.0)	0/21(0.0)	0/3(0.0)	0/3(0.0)	0/3(0.0)
Slough	0/24(0.0)	3/24(12.5)	1/21(4.8)	0/3(0.0)	1/3(33.3)	0/3(0.0)
Dermal Hemorrhage	2/24(8.3)	0/24(0.0)	0/21(0.0)	0/3(0.0)	0/3(0.0)	0/3(0.0)
Dermal Necrosis	9/24(37.5)	14/24(58.3)	20/21(95.2)	2/3(66.7)	3/3(100.0)	3/3(100.0)
Dermal Ulceration	0/24(0.0)	1/24(4.2)	0/21(0.0)	0/3(0.0)	0/3(0.0)	0/3(0.0)
Dermal Neutrophil Infiltration	22/24(91.7)	22/24(91.7)	19/21(90.5)	3/3(100.0)	2/3(66.7)	2/3(66.7)
Dermal Edema	8/24(33.3)	5/24(20.8)	0/21(0.0)	2/3(66.7)	0/3(0.0)	0/3(0.0)
Neovascularization	0/24(0.0)	0/24(0.0)	0/21(0.0)	0/3(0.0)	0/3(0.0)	0/3(0.0)
Granulation	0/24(0.0)	2/24(8.3)	1/21(4.8)	0/3(0.0)	0/3(0.0)	0/3(0.0)
Subcutaneous Hemorrhage	2/24(8.3)	0/24(0.0)	0/21(0.0)	0/3(0.0)	0/3(0.0)	0/3(0.0)
Subcutaneous Edema	16?24(66.7)	8/24(33.3)	2/21(9.5)	3/3(100.0)	0/3(0.0)	0/3(0.0)
Subcutaneous Neutrophil Infiltration	23/24(95.8)	20/24(83.3)	21/21(100.0)	2/3(66.7)	3/3(100.0)	3/3(100.0)
Subcutaneous Necrosis	9/24(37.5)	11/24(45.8)	19/21(90.5)	2/3(66.7)	3/3(100.0)	3/3(100.0)
Re-epithelialization	0/24(0.0)	0/24(0.0)	0/21(0.0)	0/3(0.0)	0/3(0.0)	0/3(0.0)
Infection	0/24(0.0)	6/24(25.0)	9/21(42.9)	0/3(0.0)	1/3(33.3)	1/3(33.3)

[1] Trypsin was applied 1 hour for animal 96-2-10.

Table A-4. Incidence of Histopathology Endpoints on Sites Dosed with 500 μL or 600 μL HD for Two Hours.

Histopathology Endpoint	HD Dose = 500 μL-hr			HD Dose = 600 μL-hr	
	Nair, Papain, and Needle Pretreatment	Nair and Trypsin Pretreatment		Nair and Trypsin Pretreatment	
	Day 5	Day 2	Day 6	Day 2	Day 6
Epidermal Necrosis	2/2(100.0)	2/2(100.0)	2/2(100.0)	2/2(100.0)	2/2(100.0)
Follicular Necrosis	2/2(100.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)
Epidermal Ulceration	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)
Epidermal Neutrophil Infiltration	1/2(50.0)	0/2(0.0)	2/2(100.0)	0/2(0.0)	0/2(0.0)
Microblister	0/2(0.0)	0/2(0.0)	1/2(50.0)	0/2(0.0)	2/2(100.0)
Slough	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)
Dermal Hemorrhage	0/2(0.0)	0/2(0.0)	2/2(100.0)	0/2(0.0)	1/2(50.0)
Dermal Necrosis	2/2(100.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)
Dermal Ulceration	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)
Dermal Neutrophil Infiltration	1/2(50.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)
Dermal Edema	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)
Neovascularization	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)
Granulation	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)
Subcutaneous Hemorrhage	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)
Subcutaneous Edema	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)
Subcutaneous Neutrophil Infiltration	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)
Subcutaneous Necrosis	2/2(100.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)
Re-epithelialization	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)
Infection	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)	0/2(0.0)

TABLE E-1. PHASE 1 HISTOLOGICAL DATA FOR DERMAL, HD-DOSED ANIMALS (N=16)

DATE	ANIMAL ID	SITE ID DORSAL	HD DOSE µL-hr	PRETX	ABRADE	STUDY DAY	EN	FN	EU	ENEUIN	MB	SLOUGH	DH	DN	DU	DNEUIN	DEDEM	NEOVAS	GRAN	SQH	SQED	SQNEUIN	SQN	RE-EPI	INFEC
4/16/96	96-13-9	A	200-2	N,TS	No	6	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		B	200-2	N,TR	No	6	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		C	200-1.5	N,TS	No	6	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
		D	200-1.5	N,TR	No	6	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
		E	200-1	N,TS	No	6	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		F	200-1	N,TR	No	6	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
4/29/96	96-13-7	A	500-2	N,TR	NE	5	1+	1+	0	0	0	0	0	1+	0	0	0	0	0	0	0	0	1-	0	0
		B	500-2	N,PA	NE	5	1+	1+	0	0	0	0	0	1+	0	0	0	0	0	0	0	0	1-	0	0
		B2	500-2	N,PA	NE	5	1+	1+	0	1	0	0	0	1+	0	1	0	0	0	0	0	0	1-	0	0
		C	500-2	N,TR	SP	5	1+	1+	0	0	0	0	0	1+	0	0	0	0	0	0	0	0	1-	0	0
		D	500-2	N,PA	SP	5	1+	1+	0	0	0	0	0	1+	0	0	0	0	0	0	0	0	1-	0	0
		E	500-2	N,TR	No	5	1+	1+	0	1+	0	0	0	0	0	1+	0	0	0	0	0	1	0	0	0
		F	500-2	N,PA	No	5	1+	1+	0	1	0	0	0	0	0	1+	0	0	0	0	0	1	0	0	0
5/9/96	96-13-7	D	500-2			15	1+	1+	1+	0	0	1+	0	0	0	1+	0	1	1+	0	0	1	1	0	0
	96-21-7		No histopath																						
5/17/96	96-21-10	A	300-2	N,TR	NE	3	1+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		B	300-2	N,TR	NE	3	1+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		C	300-2	N,TR	SP	3	1+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		D	300-2	N,TR	SP	3	1+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		E	300-2	N,TR	ESP	3	1+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		F	300-2	N,TR	ESP	3	1+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5/20/96	96-21-10	A	300-2	N,TR	NE	6	1+	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
		B	300-2	N,TR	NE	6	1+	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
		C	300-2	N,TR	SP	6	1+	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
		D	300-2	N,TR	SP	6	1+	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
		E	300-2	N,TR	ESP	6	1+	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
		F	300-2	N,TR	ESP	6	1+	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0

TABLE E-1. PHASE 1 HISTOLOGICAL DATA FOR DERMAL, HD-DOSED ANIMALS (N=16)

DATE	ANIMAL ID	SITE ID DORSAL	HD DOSE µL-hr	PRETX	ABRADE	STUDY DAY	EN	FN	EU	ENEUIN	MB	SLOUGH	DII	DN	DU	DNEUIN	DEDEM	NEOVAS	GRAN	SQII	SQED	SQNEUIN	SQN	RE-EPI	INFEC
5/24/96	96-21-10	A	300-2	N,TR	NE	9	0	0	0	1+	0	0	0	1+	0	1	0	0	0	0	0	0	0	0	0
		B	300-2	N,TR	NE	9	0	0	0	1+	0	0	0	1+	0	1	0	0	0	0	0	0	0	0	0
		C	300-2	N,TR	SP	9	0	0	0	1+	0	0	0	1+	0	1	0	0	0	0	0	0	0	0	0
		D	300-2	N,TR	SP	9	0	0	0	1+	0	0	0	1+	0	1	0	0	0	0	0	0	0	0	0
		E	300-2	N,TR	ESP	9	0	0	0	1+	0	0	0	1+	0	1	0	0	0	0	0	0	0	0	0
		F	300-2	N,TR	ESP	9	0	0	0	1+	0	0	0	1+	0	1	0	0	0	0	0	0	0	0	0
5/28/96	96-21-10	A	300-2	N,TR	NE	12	0	0	0	0	0	1+	0	1+	0	1+	0	1+	1+	0	0	0	0	1-	0
		B	300-2	N,TR	NE	12	0	0	0	0	0	1+	0	1+	0	1+	0	1+	1+	0	0	0	0	1-	0
		C	300-2	N,TR	SP	12	0	0	0	0	0	1+	0	1+	0	1+	0	1+	1+	0	0	0	0	1-	0
		D	300-2	N,TR	SP	12	0	0	0	0	0	1+	0	1+	0	1+	0	1+	1+	0	0	0	0	1-	0
		E	300-2	N,TR	ESP	12	0	0	0	0	0	1+	0	1+	0	1+	0	1+	1+	0	0	0	0	1-	0
		F	300-2	N,TR	ESP	12	0	0	0	0	0	1+	0	1+	0	1+	0	1+	1+	0	0	0	0	1-	0
6/12/96	96-34-5	A	300-2	N,TR	No	1	1+	0	0	0	0	0	1-	0	0	0	0	0	0	0	0	0	0	0	0
		B	600-2	N,TR,TS	No	1	1+	0	0	0	0	0	1-	0	0	0	0	0	0	0	0	0	0	0	0
		C	600-2	N,TR	No	1	1+	0	0	0	0	0	1-	0	0	0	0	0	0	0	0	0	0	0	0
		D	300-2	N,TR,TS	No	1	1+	1-	0	0	0	0	1-	0	0	0	0	0	0	0	0	0	0	0	0
		E	500-2	N,TR	No	1	1+	0	0	0	0	0	1-	0	0	0	0	0	0	0	0	0	0	0	0
		F	500-2	N,TR,TS	No	1	1+	0	0	0	0	0	1-	0	0	0	0	0	0	0	0	0	0	0	0
6/13/96	96-34-5	A	300-2	N,TR	No	2	1+	0	0	0	1-	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		B	600-2	N,TR,TS	No	2	1+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		C	600-2	N,TR	No	2	1+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		D	300-2	N,TR,TS	No	2	1+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		E	500-2	N,TR	No	2	1+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		F	500-2	N,TR,TS	No	2	1+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6/17/96	96-34-5	A	300-2	N,TR	No	6	1+	0	0	1+	1	0	1-	0	0	0	0	0	0	0	0	0	0	0	0
		B	600-2	N,TR,TS	No	6	1+	0	0	0	1	0	1-	0	0	0	0	0	0	0	0	0	0	0	0
		C	600-2	N,TR	No	6	1+	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		D	300-2	N,TR,TS	No	6	1+	0	0	0	1	0	1-	0	0	1	0	0	0	0	0	0	0	0	0
		E	500-2	N,TR	No	6	1+	0	0	1+	0	0	1-	0	0	0	0	0	0	0	0	0	0	0	0
		F	500-2	N,TR,TS	No	6	1+	0	0	1+	1	0	1-	0	0	0	0	0	0	0	0	0	0	0	0
6/28/96	96-34-4	F1	300-2	N,TR	No	3	1+	1-	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0
		F2	300-2	N,TR	No	3	1+	0	0	1	0	0	0	1	0	1+	0	0	0	0	0	0	0	0	0
7/1/96		D1	300-2	N,TR	No	6	1+	0	0	1+	1	0	1	0	0	1	0	0	0	0	0	0	0	1-	1-
		D2	300-2	N,TR	No	6	1+	0	0	0	0	0	0	0	0	1+	0	0	0	0	0	0	0	0	0
7/4/96		B1	300-2	N,TR	No	9	1+	0	1	0	0	1	0	1	0	1	1-	0	0	0	0	0	0	0	0
		B2	300-2	N,TR	No	9	1+	1	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1-
7/7/96		A1	300-2	N,TR	No	12	0	0	1	0	0	1	0	1	0	1+	0	0	1	0	0	0	0	1-	0
		A2	300-2	N,TR	No	12	0	0	1	0	0	1	0	1+	0	1+	0	0	1	0	0	0	0	0	0
7/10/96		E1	300-2	N,TR	No	15	0	0	1	0	0	1	0	1	0	1	0	0	1-	0	0	0	0	1	0
		E2	300-2	N,TR	No	15	0	0	0	0	0	0	0	1+	0	1+	0	0	0	0	0	0	0	0	0
7/13/96		C1	300-2	N,TR	No	18	0	0	0	0	0	0	0	0	0	0	0	1	1+	0	0	0	0	1+	0
		C2	300-2	N,TR	No	18	0	0	0	0	0	0	0	0	0	1+	0	0	1+	0	0	0	0	1+	0

TABLE E-1. PHASE 1 HISTOLOGICAL DATA FOR DERMAL, IID-DOSED ANIMALS (N=16)

DATE	ANIMAL ID	SITE ID DORSAL	IID DOSE µL-hr	PRETX	ABRADE	STUDY DAY	EN	FN	EU	ENEUIN	MB	SLOUGH	DII	DN	DU	DNEUIN	DEDEM	NEOVAS	GRAN	SQII	SQED	SQNEUIN	SQN	RE-EPI	INFEC
8/22/96	96-1-4	A	400-2	N,TR	No	3	1+	0	0	1+	0	0	0	0	0	1	1+	0	0	0	1+	1	0	0	0
		B	400-2	N,TR	No	3	1+	0	0	0	0	0	0	0	0	1-	0	0	0	0	1	1-	0	0	0
		C	400-2	N,TR	No	3	1+	0	0	0	0	0	0	0	0	1-	0	0	0	1	1	1-	0	0	0
		D	400-2	N,TR	No	3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1-	0	0	0
		E	400-2	N,TR	No	3	1-	0	0	0	0	0	0	0	0	1-	1-	0	0	0	1	1-	0	0	0
		F	400-2	N,TR	No	3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1-	0	0	0
8/25/96	96-1-4	A	400-2	N,TR	No	6	1+	0	0	1+	0	0	0	0	0	1	0	0	0	0	1-	1	0	0	0
		B	400-2	N,TR	No	6	1+	0	0	1+	0	0	0	1	0	1+	1	0	0	0	1	1+	1	0	1
		C	400-2	N,TR	No	6	1+	0	0	0	0	0	0	1	0	1-	0	0	0	0	0	1+	0	0	1
		D	400-2	N,TR	No	6	1+	0	0	1+	0	0	0	0	0	1	1	0	0	0	1-	1	0	0	0
		E	400-2	N,TR	No	6	1+	0	0	0	0	0	0	1+	0	1+	0	0	0	0	0	1+	0	0	0
		F	400-2	N,TR	No	6	1+	1+	0	0	0	0	0	0	0	1+	1	0	0	0	1	1+	0	0	0
8/27/96	96-1-4	A	400-2	N,TR	No	8	1+	0	1	0	0	0	0	1+	0	1-	0	0	0	0	0	1	0	0	0
		B	400-2	N,TR	No	8	1+	0	0	0	0	0	0	1+	0	1-	0	0	0	0	0	1+	0	0	0
		C	400-2	N,TR	No	8	1+	0	0	0	0	0	0	1+	0	1-	0	0	0	0	0	1-	1+	0	0
		D	400-2	N,TR	No	8	1+	0	0	0	0	0	0	1+	0	1-	0	0	0	0	0	1-	1+	0	0
		E	400-2	N,TR	No	8	1+	0	0	0	0	0	0	1+	0	1-	0	0	0	0	1	1-	1+	0	0
		F	400-2	N,TR	No	8	1+	0	0	0	0	0	0	1+	0	1-	0	0	0	0	0	1	1+	0	0
8/24/96	96-1-3	A	400-2	N,TR	No	3	1+	0	0	0	0	0	0	0	0	1-	1-	0	0	0	1-	1-	0	0	0
		B	400-2	N,TR	No	3	1+	1	0	0	0	0	0	0	0	1-	0	0	0	0	1	1-	0	0	0
		C	400-2	N,TR	No	3	1+	0	0	0	0	0	0	0	0	1-	0	0	0	0	1-	1-	0	0	0
		D	400-2	N,TR	No	3	1+	1	0	0	0	0	0	0	0	1-	0	0	0	0	1	1-	0	0	0
		E	400-2	N,TR	No	3	1+	0	0	1-	0	0	0	0	0	1-	1-	0	0	0	1-	1-	0	0	0
		F	400-2	N,TR	No	3	1+	0	0	1-	0	0	0	0	0	1-	1-	0	0	0	1-	1-	0	0	0
8/27/96	96-1-3	A	400-2	N,TR	No	6	1+	0	0	0	0	0	0	0	0	1-	0	0	0	0	0	1-	0	0	0
		B	400-2	N,TR	No	6	1+	1	0	0	0	0	0	0	0	1-	0	0	0	0	1-	1-	0	0	0
		C	400-2	N,TR	No	6	1+	0	0	0	0	0	0	0	0	1-	0	0	0	0	0	1-	0	0	0
		D	400-2	N,TR	No	6	1+	0	1-	0	0	0	0	1	0	1+	0	0	0	0	1-	1	0	0	0
		E	400-2	N,TR	No	6	1+	0	0	0	0	0	0	0	0	1-	0	0	0	0	0	1-	0	0	0
		F	400-2	N,TR	No	6	1+	1+	0	0	0	0	0	0	0	1+	0	0	0	0	1	1+	0	0	0
8/29/96	96-1-3	A	400-2	N,TR	No	8	1+	0	0	1+	0	0	0	1	0	1+	0	0	0	0	0	1+	1+	0	0
		B	400-2	N,TR	No	8	1+	0	0	0	0	0	0	0	0	1-	0	0	0	0	0	1	1	0	0
		C	400-2	N,TR	No	8	1+	0	0	0	0	0	0	1+	0	1	0	0	0	0	0	1	1+	0	0
		D	400-2	N,TR	No	8	1+	0	1	0	0	0	0	1+	0	1+	0	0	0	0	0	1+	1+	0	1
		E	400-2	N,TR	No	8	1+	0	0	0	0	0	0	1+	0	1+	0	0	0	0	0	1+	1+	0	1
		F	400-2	N,TR	No	8	1+	0	0	0	0	0	0	1+	0	0	0	0	0	0	0	1-	1+	0	1
8/29/96	96-3-9	A	400-2	N,TR	No	3	1+	1	0	0	0	0	0	0	0	1-	1	0	0	0	1	1-	0	0	0
		B	400-2	N,TR	No	3	1+	1	0	0	0	0	0	0	0	1+	0	0	0	0	0	1+	0	0	0
		C	400-2	N,TR	No	3	1+	0	0	0	0	0	0	0	0	1-	0	0	0	0	1-	1-	0	0	0
		D	400-2	N,TR	No	3	1+	0	0	0	0	0	0	1+	0	1	0	0	0	0	0	1	1+	0	0
		E	400-2	N,TR	No	3	1+	1	0	0	0	0	0	1+	0	1-	0	0	0	0	0	1-	1+	0	0
		F	400-2	N,TR	No	3	1+	1	0	0	0	0	0	1+	0	1-	0	0	0	0	0	1-	1+	0	0

TABLE E-1. PHASE 1 HISTOLOGICAL DATA FOR DERMAL, HD-DOSED ANIMALS (N=16)

DATE	ANIMAL ID	SITE ID DORSAL	HD DOSE µL-hr	PRETX	ABRADE	STUDY DAY	EN	FN	EU	ENEUIN	MB	SLOUGH	DH	DN	DU	DNEUIN	DEDEM	NEOVAS	GRAN	SQH	SQED	SQNEUIN	SQN	RE EPI	INFEC
9/1/96	96-3-9	A	400-2	N,TR	No	6	1+	1	1	1+	0	0	0	1+	1	1+	0	0	0	0	0	0	1+	0	0
		B	400-2	N,TR	No	6	1+	1	0	1+	0	0	0	0	0	1+	0	0	0	0	0	0	0	0	1
		C	400-2	N,TR	No	6	1+	0	0	1+	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
		D	400-2	N,TR	No	6	1+	0	0	1+	0	0	0	1+	0	1+	1+	0	0	0	0	1+	1+	0	1
		E	400-2	N,TR	No	6	1+	0	0	1+	0	0	0	1+	0	1+	1+	0	0	0	0	1+	1+	0	1
		F	400-2	N,TR	No	6	1+	1+	0	0	0	0	0	1+	0	1	0	0	0	0	1	1	1+	0	0
9/3/96	96-3-9	A	400-2	N,TR	No	8	1+	1+	0	1+	0	0	0	1+	0	1+	0	0	0	0	1+	1+	1+	0	0
		B	400-2	N,TR	No	8	1+	1+	0	1+	0	0	0	1+	0	1	0	0	0	0	0	1	1+	0	0
		C	400-2	N,TR	No	8	1+	1+	1	1+	0	0	0	1+	0	1	0	0	0	0	0	1	1+	0	1
		D	400-2	N,TR	No	8	1+	1+	1	1+	0	0	0	1+	0	1	0	0	0	0	0	1	1+	0	1
		E	400-2	N,TR	No	8	1+	1+	1	1+	0	1-	0	1+	0	1	0	0	0	0	0	1	1+	0	1
		F	400-2	N,TR	No	8	1+	1+	0	1+	0	0	0	1+	0	1+	0	0	0	0	0	1+	1+	0	1
8/31/96	96-2-10	A	400-2	N,TR	No	3	1+	0	0	0	0	0	0	1	0	1-	0	0	0	0	0	1-	1	0	0
		B	400-2	N,TR	No	3	1+	0	0	0	0	0	0	1	0	1-	1	0	0	0	1	1-	1	0	0
		C	400-2	N,TR	No	3	1+	0	0	0	0	0	0	1	0	1-	1	0	0	0	1	1-	1	0	0
		D	400-2	N,TR	No	3	1+	0	0	0	0	0	0	1	0	1-	1	0	0	0	1	1-	1	0	0
		E	400-2	N,TR	No	3	1+	0	0	0	0	0	0	1	0	1	1	0	0	0	1	1-	1	0	0
		F	400-2	N,TR	No	3	1+	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0
9/3/96	96-2-10	A	400-2	N,TR	No	6	1+	1+	1	1+	0	1-	0	1+	0	1+	0	0	0	0	0	0	1+	0	1
		B	400-2	N,TR	No	6	1+	1+	0	1-	0	0	0	1+	0	1-	0	0	0	0	0	1-	1+	0	0
		C	400-2	N,TR	No	6	1+	1+	1-	0	0	1-	0	1+	0	1-	0	0	0	0	0	1	1+	0	0
		D	400-2	N,TR	No	6	1+	1+	1	1-	0	1	0	1+	0	1-	0	0	0	0	0	1-	1+	0	1
		E	400-2	N,TR	No	6	1+	0	0	0	0	0	0	1+	0	0	0	0	0	1-	0	0	1-	1+	0	0
		F	400-2	N,TR	No	6	1+	0	0	0	0	0	0	1+	0	0	0	0	0	0	0	0	1-	1+	0	0
9/5/96	96-2-10	A	400-2	N,TR	No	8	1+	1+	0	0	0	0	0	1+	0	0	0	0	0	0	0	1	1+	0	0
		B	400-2	N,TR	No	8	1+	1+	0	1+	0	0	0	1+	0	1	0	0	0	0	0	1	1+	0	0
		C	400-2	N,TR	No	8	1+	1+	1	1+	0	0	0	1+	0	1	0	0	0	0	0	1	1+	0	1
		D	400-2	N,TR	No	8	1+	1+	1	1+	0	0	0	1+	0	1	0	0	0	0	0	1	1+	0	1
		E	400-2	N,TR	No	8	1+	1+	1	1+	0	0	0	1+	0	1	0	0	1	0	0	1-	1+	0	1
		F	400-2	N,TR	No	8	1+	1+	1	0	0	0	0	1+	0	0	0	0	0	0	0	1	1+	0	0
9/7/96	96-5-8	A	400-2	N,TR	No	3	1+	1+	0	0	0	0	1-	1+	0	1-	0	0	0	0	0	0	1+	0	0
		B	400-2	N	No	3	1+	1+	0	0	0	0	1-	1+	0	1-	0	0	0	0	0	0	1+	0	0
		C	400-2	N,TR	No	3	1+	1+	0	0	0	0	1	1+	0	1	0	0	0	1	0	1	1+	0	0
		D	400-2	N	No	3	1+	1+	0	0	0	0	0	1+	0	1-	0	0	0	0	0	1-	1+	0	0
		E	400-2	N,TR	No	3	1+	1+	0	0	0	0	0	1+	0	1-	0	0	0	0	0	1-	1+	0	0
		F	400-2	N	No	3	1+	1+	0	0	0	0	0	1+	0	1-	0	0	0	1	1	1-	1+	0	0
9/10/96	96-5-8	A	400-2	N,TR	No	6	1+	1+	0	1	0	0	0	1+	0	1	0	0	1-	0	0	1-	1+	0	0
		B	400-2	N	No	6	1+	1+	0	1	0	0	0	1+	0	1	0	0	1-	0	0	1-	1+	0	0
		C	400-2	N,TR	No	6	1+	1+	0	0	0	0	0	1+	0	1-	0	0	0	0	0	1	1+	0	0
		D	400-2	N	No	6	1+	1+	0	1+	0	0	0	1+	0	1-	0	0	0	0	0	1	1+	0	0
		E	400-2	N,TR	No	6	1+	1+	0	0	0	0	0	1+	0	1-	0	0	0	0	0	1	1+	0	0
		F	400-2	N	No	6	1+	1+	0	0	0	0	0	1+	0	1-	0	0	0	0	0	1	1+	0	0

TABLE E-1. PHASE I HISTOLOGICAL DATA FOR DERMAL, HD-DOSED ANIMALS (N=16)

DATE	ANIMAL ID	SITE ID DORSAL	HD DOSE µL-hr	PRETX	ABRADE	STUDY DAY	EN	FN	EU	ENEUIN	MB	SLOUGH	DH	DN	DU	DNEUIN	DEDEM	NEOVAS	GRAN	SQH	SQED	SQNEUIN	SQN	RE-EPI	INFEC
9/18/96	96-10-12	A	400-2	N	No	2	1+	0	0	1-	0	0	0	0	0	0	0	0	0	1-	1	1-	0	0	0
		B	400-2	N	No	2	1+	0	0	0	0	0	0	0	0	1-	0	0	0	0	1	1-	0	0	0
		C	400-2	N	No	2	1+	0	0	0	0	0	0	0	0	1-	0	0	0	1-	1	1-	0	0	0
		D	400-2	N	No	2	1+	0	0	0	0	0	0	0	0	1-	0	0	0	1-	1	1-	0	0	0
		E	400-2	N	No	2	1+	0	0	0	0	0	0	1	0	0	1	0	0	1-	1+	0	1	0	0
		F	400-2	N	No	2	1+	1+	0	0	0	0	0	1	0	1+	0	0	0	0	1+	1+	0	0	0
9/19/96	96-10-11	A	400-2	N	No	2	1+	1+	0	0	0	0	0	1+	0	1-	1	0	0	0	1	1-	0	0	0
		B	400-2	N	No	2	1+	1+	0	0	0	0	0	1+	0	1-	1	0	0	0	1	1-	0	0	0
		C	400-2	N	No	2	1+	1+	0	0	0	0	0	1+	0	1-	1	0	0	0	1	1-	0	0	0
		D	400-2	N	No	2	1+	1+	0	0	0	0	0	1	0	1-	0	0	0	0	1-	1-	0	0	0
		E	400-2	N	No	2	0	0	0	0	0	0	0	0	0	1-	0	0	0	0	1-	1-	0	0	0
		F	400-2	N	No	2	1+	1+	0	0	0	0	0	1+	0	1-	1	0	0	0	1-	1-	0	0	0
9/25/96	96-11-8	A	400-2	N	No	2	1+	0	1-	0	0	1-	0	0	0	1-	0	0	0	1-	1-	1-	0	0	0
		B	400-2	N	No	2	1+	0	0	0	0	0	0	0	0	1-	0	0	0	1-	1-	1-	0	0	0
		C	400-2	N	No	2	1+	1+	0	0	0	0	0	0	0	1-	0	0	0	0	0	0	0	0	0
		D	400-2	N	No	2	1+	0	0	0	0	0	0	0	0	1-	0	0	0	1-	1-	1-	0	0	0
		E	400-2	N	No	2	1+	0	0	0	0	0	0	0	0	1-	0	0	0	1-	1-	1-	0	0	0
		F	400-2	N	No	2	1+	0	0	0	0	0	0	0	0	1-	0	0	0	1-	1-	1-	0	0	0
10/9/96	96-11-8	A	400-2	N	No	16	0	0	0	0	0	1+	0	0	0	1+	0	0	1+	0	0	1+	1+	0	0
		B	400-2	N	No	16	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		C	400-2	N	No	16	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		D	400-2	N	No	16	0	0	0	0	0	1+	0	0	0	1+	0	0	1+	0	0	1+	1+	0	0
		E	400-2	N	No	16	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		F	400-2	N	No	16	0	0	0	0	0	1+	0	0	0	0	1-	1+	1+	0	0	0	0	1+	0
9/26/96	96-11-11	A	400-2	N	No	2	1+	1+	0	0	0	0	0	0	0	1-	0	0	0	1-	1	1-	0	0	0
		B	400-2	N	No	2	1+	1+	0	0	0	0	0	0	0	1-	0	0	0	1-	1	1-	0	0	0
		C	400-2	N	No	2	1+	1+	0	0	0	0	0	0	0	1-	0	0	0	1-	1	1-	0	0	0
		D	400-2	N	No	2	1+	1+	0	0	0	0	0	0	0	1-	0	0	0	1-	1	1-	0	0	0
		E	400-2	N	No	2	1+	1+	0	0	0	0	0	0	0	1-	0	0	0	1-	1	1-	0	0	0
		F	400-2	N	No	2	1+	1+	0	0	0	0	0	0	0	1-	0	0	0	1-	1	1-	0	0	0
10/10/96	96-11-11	A	400-2	N	No	16	0	0	0	0	0	1+	0	0	0	1+	0	0	1+	0	0	1+	1+	0	0
		B	400-2	N	No	16	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		C	400-2	N	No	16	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		D	400-2	N	No	16	0	0	0	0	0	1+	0	0	0	1+	0	0	1+	0	0	1+	1+	0	0
		E	400-2	N	No	16	0	0	0	0	0	1+	0	0	0	1+	0	0	1+	0	0	1+	1+	0	0
		F	400-2	N	No	16	0	0	0	0	0	1+	0	0	0	1+	0	0	1+	0	0	1+	1+	0	0
10/17/96	96-18-8	A	400-2	N	No	2	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
		B	400-2	N	No	2	1+	1+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		C	400-2	N	No	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		D	400-2	N	No	2	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		E	400-2	N	No	2	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		F	400-2	N	No	2	1+	1+	0	0	0	0	0	1+	0	0	0	0	0	0	0	0	1+	0	0

TABLE E-1. PHASE 1 HISTOLOGICAL DATA FOR DERMAL, HD-DOSED ANIMALS (N=16)

DATE	ANIMAL ID	SITE ID DORSAL	HD DOSE µL-hr	PRETX	ABRADE	STUDY DAY	EN	FN	EU	ENEUIN	MB	SLOUGH	DH	DN	DU	DNEUIN	DEDEM	NEOVAS	GRAN	SQH	SQED	SQNEUIN	SQN	RE-EPI	INFEC
10/24/96	96-18-8	A	400-2	N	No	9	NA	NA	1	1	0	1+	1	1+	0	1	1	1-	1	0	0	1	1	1+	0
		B	400-2	N	No	9	1+	1	0	1+	0	0	0	0	0	1+	0	0	1-	0	0	1+	0	1	0
		C	400-2	N	No	9	0	0	1+	0	0	1+	0	0	0	1+	0	0	1+	0	0	1+	0	0	0
		D	400-2	N	No	9	0	0	0	0	0	1+	0	0	1	1+	0	0	1+	0	0	1+	0	1+	0
		E	400-2	N	No	9	NA	NA	1	1	0	1+	0	1+	0	1+	1	1-	1+	0	0	1+	1	1+	0
		F	400-2	N	No	9	NA	NA	1+	1	0	1+	1	1+	0	1+	1	1-	1	0	0	1+	1	1+	0
10/31/96	96-18-8	A	400-2	N	No	16	NA	NA	1-	0	0	1-	0	1	0	1	0	0	1+	0	0	1	1	1+	0
		B	400-2	N	No	16	0	0	0	0	0	0	0	0	0	1-	0	0	1-	0	0	1-	0	1	0
		C	400-2	N	No	16	0	0	0	0	0	0	0	0	0	1-	0	0	1+	0	0	1+	0	1	0
		D	400-2	N	No	16	0	0	1-	0	0	0	0	0	0	1-	0	0	1-	0	0	1-	0	1	0
		E	400-2	N	No	16	0	0	0	0	0	0	0	0	0	1-	0	0	1+	0	0	1-	0	1+	0
		F	400-2	N	No	16	0	0	0	0	0	0	0	0	0	1-	0	1-	1+	0	0	1-	0	1+	0
11/7/96	96-18-8	A	400-2	N	No	23	NA	NA	1+	1+	0	1+	0	0	1+	1+	0	0	1+	0	0	1+	0	1	0
		B	400-2	N	No	23	NA	NA	1+	0	0	1+	0	0	1+	0	0	0	1	0	0	0	0	1	0
		C	400-2	N	No	23	NA	NA	1+	1	0	1+	1-	0	1+	1	0	0	1+	1-	0	1	0	1-	0
		D	400-2	N	No	23	1+	NA	1+	1-	0	1	1-	0	1+	1-	0	0	1+	1-	0	1-	0	1-	0
		E	400-2	N	No	23	NA	NA	1+	1	0	1+	0	0	1+	1	0	0	1	0	0	1	0	1	0
		F	400-2	N	No	23	NA	NA	1+	1	0	1+	0	0	1+	1	0	0	1	0	0	1	0	1	0

KEY

EN = Epidermal Necrosis
FN = Follicular Necrosis
EU = Epidermal Ulceration
ENEUIN = Epidermal Neutrophil Infiltration
MB = Microblister
DH = Dermal Hemorrhage
DN = Dermal Necrosis
DU = Dermal Ulceration
DNEUIN = Dermal Neutrophil Infiltration
DEDEM = Dermal Edema
NEOVAS = Neovascularization
GRAN = Granulation
SQH = Subcutaneous Hemorrhage
SQED = Subcutaneous Edema
SQNEUIN = Subcutaneous Neutrophil Infiltration
SQN = Subcutaneous Necrosis
RE-EPI = Re-epithelialization
INFEC = Infection

Each category is given the following grading system.

0 = Absence
1 = Present
1+ = Present but greater
1- = Present but lessor
NA = Unknown
TS = Tape Stripping

PA = Papain
SP = Sandpaper
N = Nair
TR = Trypsin
ESP = Emery Sandpaper
NE = Needle

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PHASE 1. CLINICAL OBSERVATIONS DATA FOR DERMAL HD-DOSED ANIMALS (N=16)

Animal#	Dose Date	Obs Date	Site	Dose	Size LxW (mm)	Exudate	Erythema	Edema	Necrosis	Eschar	General Impression	Epithelialization	Inflammation	Granulation	Contraction	Infection	Rejection	Adherence	Durability	Vascularization
96-13-7	4/24/96	4/25/96	A	500 uL	40x55	1.25	4.25	2	NA	2	4.25	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	500 uL	38x58	1	5	2.25	NA	2	4	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	500 uL	40x59	1.25	4.25	2	NA	2	4	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	500 uL	41x56	0.75	4.25	2	NA	1	3.75	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	500 uL	39x62	0	3.75	0	NA	0	3	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	500 uL	36x60	0	3.75	1.75	NA	0	3	NA	NA	NA						
96-13-7	4/24/96	4/26/96	A	500 uL	NA	1.75	4	1	NA	NA	3.25	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	500 uL	NA	1.25	4	0	NA	NA	2.75	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	500 uL	NA	1.25	4	1	NA	NA	3	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	500 uL	NA	0.75	4	0	NA	NA	2	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	500 uL	NA	0	3.25	0	NA	NA	1.75	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	500 uL	NA	0	3	0	NA	NA	1.75	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-13-7	4/24/96	4/27/96	A	500 uL	NA	2	5	2	3	0	4	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	500 uL	NA	2	5	2	3	0	4	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	500 uL	NA	2	5	3	3	0	4	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	500 uL	NA	3	5	0	2	0	4	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	500 uL	NA	0	3	0	0	0	1	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	500 uL	NA	0	3	0	0	0	1	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-13-7	4/24/96	4/28/96	A	500 uL	NA	2	1.25	0	1	0	3.75	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	500 uL	NA	2	2	1	2	0	4	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	500 uL	NA	3	2	1	3	0	4.25	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	500 uL	NA	3.25	2	0.75	3.25	0	5	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	500 uL	NA	0	1.25	0	0	0	1.75	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	500 uL	NA	0	2	0	0	0	2	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-13-7	4/24/96	4/29/96	A	500 uL	39x48	3	1	1	1	0	4	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	500 uL	43x50	2	5	2.25	2	0	4.25	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	500 uL	39x53	3	5	2.75	2.25	0	4.25	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	500 uL	36x52	3	5	2.75	2.25	0	5	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	500 uL	40x53	0.75	1.25	0	0	0	2	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	500 uL	45x60	0.75	1.25	0	0	0	2	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-13-7	4/24/96	4/30/96	A	500 uL	NA	3	1	0	2.25	0	4.75	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	500 uL	NA	3	2.25	0	2	0	4.25	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	500 uL	NA	3	1	1.75	2.5	0	5	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	500 uL	NA	3	1	2	3	0	4.75	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	500 uL	NA	0.25	1.25	0	0	0	2	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	500 uL	NA	0.75	1.25	0	0	0	2	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-13-7	4/24/96	5/1/96	A	500 uL	NA	3.25	5	1	3.25	0	4	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	500 uL	NA	3.25	4.75	0.75	2.25	0	4	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	500 uL	NA	3.25	5	1.25	3.25	0	4.25	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	500 uL	NA	3.25	5	1	2.75	0	4.25	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	500 uL	NA	2	2.75	0.75	1	0	3	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	500 uL	NA	2	3	1	1.25	0	3	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-13-7	4/24/96	5/2/96	A	500 uL	NA	1	4	0.75	2	0	4	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	500 uL	NA	1	4	0	2	0	4	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	500 uL	NA	1	4	1	2	0	4	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	500 uL	NA	1.25	4.25	1	2	0	4	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	500 uL	NA	1	3	0	1.25	0	3	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	500 uL	NA	1	3.75	1	1.75	0	3.25	NA	NA	NA	NA	NA	NA	NA	NA	NA

PHASE 1. CLINICAL OBSERVATIONS DATA FOR DERMAL HD-DOSED ANIMALS (N=16)

Animal#	Dose Date	Obs Date	Site	Dose	Size LxW (mm)	Exudate	Erythema	Edema	Necrosis	Eschar	General Impression	Epithelialization	Inflammation	Granulation	Contraction	Infection	Rejection	Adherence	Durability	Vascularization
96-13-7	4/24/96	5/3/96	A	500 uL	32x47	3.25	5	2	3	0	4.75	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	500 uL	36x45	3.5	5	2.25	3	0	4.75	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	500 uL	31x51	3.25	5	2	3	0	5	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	500 uL	36x48	3.25	5	3	3	0	5	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	500 uL	39x57	3	5	1	2	0	4	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	500 uL	40x53	3	5	1	2	0	4	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-13-7	4/24/96	5/8/96	A	500 uL	37x42	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	500 uL	33x43	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	500 uL	29x47	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	500 uL	43x41	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	500 uL	30x47	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	500 uL	30x44	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-21-7	5/6/96	5/7/96	A	300 uL	33x45	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	300 uL	34x47	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	300 uL	31x39	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	300 uL	35x57	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	300 uL	35x43	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	300 uL	37x58	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-21-7	5/6/96	5/9/96	A	300 uL	41x44	1	5	0	2	0	4.25	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	300 uL	39x45	3	4.25	1	1	0	4	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	300 uL	38x44	3.25	5	1	2.25	0	4	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	300 uL	42x50	2.75	5	3	2	0	4.25	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	300 uL	44x37	2	4.75	4	1.25	0	4	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	300 uL	42x47	3	5	2	3	0	4.75	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-21-7	5/6/96	5/12/96	A	300 uL	36x42	0	5	1	2	0	4.75	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	300 uL	35x42	0	5	1	1.25	0	4.75	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	300 uL	38x40	0	5	1	2.25	0	4.75	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	300 uL	36x43	0	5	1	2	0	4.75	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	300 uL	40x38	0	5	5	2.75	0	4.25	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	300 uL	34x44	0	5	4	2.75	0	4.75	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-21-7	5/6/96	5/16/96	A	300 uL	31x38	0	5	1	3	0.75	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	300 uL	34x42	0	5	2	3	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	300 uL	37x37	1	5	2	3	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	300 uL	35x40	0.75	5	2	3	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	300 uL	36x35	0	5	2	3	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	300 uL	34x45	0	5	2	3	0.75	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-21-7	5/6/96	5/23/96	A	300 uL	29x30	0	5	0	3	0	5	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	300 uL	24x34	0	5	0	3	0	5	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	300 uL	38x33	0	5	1	3	0	5	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	300 uL	33x33	0	5	1	3	0	5	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	300 uL	38x32	0	5	1	3	0	5	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	300 uL	33x34	0	5	1	3	0	5	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-21-7	5/6/96	5/26/96	A	300 uL	26x34	1	5	1	3	0	5	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	300 uL	28x31	0	5	0	3	0	5	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	300 uL	37x34	0	5	1	3	0	5	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	300 uL	38x36	1	5	2	3	0	5	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	300 uL	35x38	0	5	0	3	0	5	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	300 uL	37x35	0	5	0	3	0	5	NA	NA	NA	NA	NA	NA	NA	NA	NA

PHASE 1. CLINICAL OBSERVATIONS DATA FOR DERMAL HD-DOSED ANIMALS (N=16)

Animal#	Dose Date	Obs Date	Site	Dose	Size LxW (mm)	Exudate	Erythema	Edema	Necrosis	Eschar	General Impression	Epithelialization	Inflammation	Granulation	Contraction	Infection	Rejection	Adherence	Durability	Vascularization
96-21-10	5/14/96	5/17/96	A	300 uL	40x45	0	2	0	0	0	2	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	300 uL	39x48	0	2	1	0	0	2	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	300 uL	40x48	0	2	0	0	0	2	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	300 uL	40x49	0	2	2	0	0	2	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	300 uL	38x48	0	2	1	0	0	2	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	300 uL	37x45	0	2	1	0	0	2	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-21-10	5/14/96	5/20/96	A	300 uL	40x49	0	2	1	0	0	2	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	300 uL	38x47	1	2	2	0	0	2	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	300 uL	37x52	0	3	1	1	0	3	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	300 uL	35x48	1	3	2	1	1	3	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	300 uL	40x52	0	3	2	1	0	3	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	300 uL	35x45	1	3	2	1	1	3	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-21-10	5/14/96	5/24/96	A	300 uL	40x43	2	5	2	3	NA	5	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	300 uL	35x40	0	5	2.25	3	NA	4	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	300 uL	35x45	2	5	1	3	NA	5	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	300 uL	34x43	1.25	5	2	3	NA	5	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	300 uL	35x45	1	5	2	3	NA	5	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	300 uL	34x49	1.25	5	2	3	NA	5	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-21-10	5/14/96	5/28/96	A	300 uL	30x35	1	5	0	3	0	5	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	300 uL	37x40	0	5	0	3	0	5	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	300 uL	37x39	1	5	1	3	0	5	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	300 uL	40x40	1	5	0	3	0	5	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	300 uL	32x40	0	5	1	3	0	5	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	300 uL	33x41	1	5	0	3	0	5	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-34-5	6/11/96	6/12/96	A	300 uL	46x39	0	4.25	1	0	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	600 uL	NAx50	1	5	2	1	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	600 uL	52x45	1	5	1	0.75	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	300 uL	NAx38	0	4	1	0	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	500 uL	42x41	0	4.25	0	0	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	500 uL	NAx67	1	5	4	1.25	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-34-5	6/11/96	6/13/96	A	300 uL	45x42	0	4.25	0	0	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	600 uL	57x42	0.75	5	4	0.75	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	600 uL	49x49	0.75	5	1	1	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	300 uL	40x30	0	4.25	3	0	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	500 uL	47x45	0	4.25	0	0	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	500 uL	58x40	0	4.75	1	0	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-34-5	6/11/96	6/17/96	A	300 uL	36x41	0	4.5	1	0.75	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	600 uL	45x48	1	5	2	1.25	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	600 uL	44x50	1	5	0.75	0.75	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	300 uL	30x35	0	4	0	0	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	500 uL	41x42	0.75	4.25	1	0.75	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	500 uL	40x53	0.75	5	2.25	0.75	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-34-4	6/25/96	7/13/96	A	300 uL	33x34	0	0	0	3.25	3.25	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	300 uL	25x25	0	0	0	3.25	3.25	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	300 uL	30x34	0	0	0	3.25	3.25	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	300 uL	25x32	2	0	0	3.25	3.5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	300 uL	NA	0	0	0	3	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	300 uL	20x31	3	0	0	1.75	4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

PHASE 1. CLINICAL OBSERVATIONS DATA FOR DERMAL HD-DOSED ANIMALS (N=18)

Animal#	Dose Date	Obs Date	Site	Dose	Size LxW (mm)	Exudate	Erythema	Edema	Necrosis	Eschar	General Impression	Epithelialization	Inflammation	Granulation	Contraction	Infection	Rejection	Adherence	Durability	Vascularization
96-1-4	8/19/96	8/22/96	A	400 uL	45x44	0	5	2.25	1.25	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	400 uL	46x42	0	4.75	3.5	1	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	400 uL	47x47	0	4.75	2.75	0.75	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	400 uL	49x42	0	5	3.5	1	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	400 uL	42x46	0	5	3.5	1	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	400 uL	55x41	0	4.75	3	0.75	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-1-4	8/19/96	8/25/96	A	400 uL	45x42	3	2	2	5	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	400 uL	54x44	1	2.5	4	5	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	400 uL	52x42	3	2	2.5	5	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	400 uL	58x44	1	2.5	4	5	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	400 uL	52x44	2.25	2	4	4.75	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	400 uL	70x43	1.5	3	4	5	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-1-4	8/19/96	8/27/96	A	400 uL	46x40	0	0.75	0	4	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	400 uL	42x43	0	0.75	0	4	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	400 uL	49x44	0	0.75	0	4	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	400 uL	52x44	0	2	0	4	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	400 uL	50x42	0	1	0	4	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	400 uL	57x40	0	2	0	4	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-1-3	8/21/96	8/24/96	A	400 uL	45x40	0	4.25	0.75	0.75	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	400 uL	45x47	0	4.75	1	1	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	400 uL	50x40	0	4.25	1	0.75	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	400 uL	47x43	0	4.5	1	0.75	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	400 uL	45x41	0	4.5	1.25	1	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	400 uL	48x42	0	4.5	1.25	0.75	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-1-3	8/21/96	8/27/96	A	400 uL	49x41	0	5	1	0.75	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	400 uL	49x53	0	5	1	0.75	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	400 uL	49x47	0.5	5	1	0.75	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	400 uL	45x55	0	5	1	3	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	400 uL	52x50	0.5	5	3	3.5	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	400 uL	50x50	0.5	5	3	3.75	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-1-3	8/21/96	8/29/96	A	400 uL	52x56	0	2.25	1	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	400 uL	49x55	0	2	1	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	400 uL	60x48	0.5	2.75	1	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	400 uL	57x53	1	3	1	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	400 uL	50x52	2	1	1	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	400 uL	50x54	1	2	2	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-3-9	8/26/96	8/29/96	A	400 uL	41x54	0	4	1	0	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	400 uL	57x43	0	4.25	1	1	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	400 uL	43x55	0	4	1	0.5	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	400 uL	55x44	0	4.25	2.25	1	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	400 uL	44x55	0	4.25	2	0.5	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	400 uL	54x42	0	4.25	2.25	0.75	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-3-9	8/26/96	9/1/96	A	400 uL	45x50	0.75	1	1	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	400 uL	45x54	1	1	1	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	400 uL	47x57	0.75	1	1	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	400 uL	46x52	1	1	1	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	400 uL	45x58	1	2	2	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	400 uL	47x63	1	2	2	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

PHASE 1. CLINICAL OBSERVATIONS DATA FOR DERMAL HD-DOSED ANIMALS (N=16)

Animal#	Dose Date	Obs Date	Site	Dose	Size LxW (mm)	Exudate	Erythema	Edema	Necrosis	Eschar	General Impression	Epithelialization	Inflammation	Granulation	Contraction	Infection	Rejection	Adherance	Durability	Vascularization
96-3-9	8/26/96	9/3/96	A	400 uL	47x59	0	1	0	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	400 uL	44x48	1	1	0	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	400 uL	43x54	0	2	0	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	400 uL	45x49	0.75	2	1	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	400 uL	40x54	0	2.25	0	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	400 uL	40x56	0	2.5	1	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-2-10	8/28/96	8/31/96	A	400 uL	38x51	3	5	0.75	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	400 uL	38x54	2.25	5	0.75	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	400 uL	42x53	1	5	0.75	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	400 uL	43x56	2	5	0.75	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	400 uL	39x52	0	5	2	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	400 uL	38x57	1	5	0.75	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-2-10	8/28/96	9/3/96	A	400 uL	35x42	0	0.75	0	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	400 uL	34x42	0	0.75	0	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	400 uL	40x54	0.5	0.75	0	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	400 uL	43x51	0.5	0.75	0	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	400 uL	40x55	0	0.75	0	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	400 uL	43x50	0	0.75	0	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-2-10	8/28/96	9/5/96	A	400 uL	32x40	0	1	0	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	400 uL	32x45	0	0.75	0	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	400 uL	40x53	0	1.5	0	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	400 uL	44x48	1	2	0	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	400 uL	43x57	1	2	0	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	400 uL	42x45	1	2.5	0	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-5-8	9/4/96	9/7/96	A	400 uL	42x41	0.75	5	1	1	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	400 uL	39x47	2	5.5	1	1	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	400 uL	41x37	0.75	5.25	1	1.25	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	400 uL	39x43	0.5	5.25	2	1.25	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	400 uL	44x40	0	5.25	1	1.5	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	400 uL	42x40	0.5	5.25	2	1.5	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-5-8	9/4/96	9/10/96	A	400 uL	41x42	0	5	0.75	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	400 uL	45x45	0	5	0.75	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	400 uL	44x40	0	5	1	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	400 uL	47x45	0	5	1	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	400 uL	50x45	0	5	2	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	400 uL	45x45	0	5	2	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-10-12	9/16/96	9/18/96	A	400 uL	43x44	0	5	1	1	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	400 uL	40x46	0	4.75	1	0.75	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	400 uL	42x43	0.75	4.75	2	0.75	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	400 uL	39x50	0	5.5	2	1.5	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	400 uL	38x42	0	5	2.5	1.25	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	400 uL	42x46	0	5.25	2.5	1.25	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-10-11	9/17/96	9/19/96	A	400 uL	40x44	0	4.25	1	0.75	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	400 uL	39x46	0	4.25	0.75	0	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	400 uL	39x50	0	4.75	0.75	1	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	400 uL	39x45	0	4.25	0.75	0.75	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	400 uL	42x47	0	4.75	2	1	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	400 uL	39x48	0	4.75	2	1	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

PHASE 1. CLINICAL OBSERVATIONS DATA FOR DERMAL HD-DOSED ANIMALS (N=16)

Animal#	Dose Date	Obs Date	Site	Dose	Size LxW (mm)	Exudate	Erythema	Edema	Necrosis	Eschar	General Impression	Epithelialization	Inflammation	Granulation	Contraction	Infection	Rejection	Adherence	Durability	Vascularization
96-11-8	9/23/96	9/25/96	A	400 uL	39x45	1	4.5	2	1	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	400 uL	38x46	0	4.5	1.75	1	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	400 uL	43x48	0	5	1.75	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	400 uL	43x44	0	5	2	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	400 uL	35x45	0	5	2	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	400 uL	35x47	0	5	2	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-11-8	9/23/96	10/9/96	A	400 uL	NA	0	NA	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	400 uL	NA	0	1.25	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	400 uL	NA	0	NA	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	400 uL	NA	0	NA	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	400 uL	NA	0	1.25	0	1 scab	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	400 uL	NA	0	NA	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-11-11	9/24/96	9/26/96	A	400 uL	42x44	0	5	2	3	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	400 uL	37x48	0	3.25	1.75	3	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	400 uL	37x43	0	4.5	1	1.5	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	400 uL	33x48	0	5	2	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	400 uL	37x46	0	5	2	3.5	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	400 uL	33x43	0	5	2	4	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-18-8	10/15/96	10/17/96	A	400 uL	40x45	1.25	5	3	1	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			B	400 uL	40x48	1.5	5	3	1	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			C	400 uL	40x48	0.5	5	3	1	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			D	400 uL	39x47	0	5	3	0.75	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			E	400 uL	37x45	0.5	5	3	1	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			F	400 uL	37x50	0	4.5	2.75	1	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
96-18-8	10/15/96	10/24/96	A	400 uL	54x40	1	NA	NA	NA	NA	NA	1.25	2	2	2	1	1	NA	1	1
			B	400 uL	45x60	1	NA	NA	NA	NA	NA	1	1	3	2	1	1	NA	0	0
			C	400 uL	44x47	1	NA	NA	NA	NA	NA	2.75	1	1	2	1.25	3	NA	2	3
			D	400 uL	NA	1	NA	NA	NA	NA	NA	2	1	2	2	1.5	1	NA	1	1
			E	400 uL	50x52	1	NA	NA	NA	NA	NA	3	1.25	1.25	2	1.25	2.25	NA	2	3
			F	400 uL	NA	1	NA	NA	NA	NA	NA	3	3	1.75	2	1.5	3	NA	2	2
96-18-8	10/15/96	10/31/96	A	400 uL	32x30	0.75	NA	NA	NA	NA	NA	0	1.5	1	1	0	0	3.5	3.25	3.5
			B	400 uL	31x34	1	NA	NA	NA	NA	NA	0	0	1	1	0	0	0	0	0
			C	400 uL	34x34	0	NA	NA	NA	NA	NA	0	3.5	1	1	0	0	0.25	0.25	0
			D	400 uL	36x32	0	NA	NA	NA	NA	NA	0	1	1	1	0	0	0.25	0.25	0
			E	400 uL	34x24	0	NA	NA	NA	NA	NA	0	3.5	1	1	0	0	0.25	0.25	0
			F	400 uL	47x36	0	NA	NA	NA	NA	NA	0	1.25	1	1	1	1	4	4	0
96-18-8	10/15/96	11/7/96	A	400 uL	25x20	0	NA	NA	NA	NA	NA	0	2	1	1	0	0	3.5	3.5	0
			B	400 uL	40x47	0	NA	NA	NA	NA	NA	0	0	1	1	0	0	0	0	0
			C	400 uL	34x19	0	NA	NA	NA	NA	NA	0	1.75	1	1	1	0	0	0	0
			D	400 uL	37x27	0	NA	NA	NA	NA	NA	0	3	1	1	0	0	0	0	0
			E	400 uL	37x11	0	NA	NA	NA	NA	NA	0	2	1	1	1	0	0	0	0
			F	400 uL	20x28	0	NA	NA	NA	NA	NA	0	3	1	1	0	0	4	4	0
96-18-8	10/15/96	11/14/96	A	400 uL	10x12	0	NA	NA	NA	NA	NA	0	0	3	healed	0	0	0	0	0
			B	400 uL	NA	0	NA	NA	NA	NA	NA	0	0	3	healed	0	NA	NA	0	0
			C	400 uL	NA	0	NA	NA	NA	NA	NA	0	0	3	healed	0	0	0	0	0
			D	400 uL	7x20	0.5	NA	NA	NA	NA	NA	1	0	3	<1/3 healed	0	NA	NA	0	0
			E	400 uL	NA	0	NA	NA	NA	NA	NA	0	0	3	healed	0	0	0	0	0
			F	400 uL	7x26	0.25	NA	NA	NA	NA	NA	0.75	0	3	<1/3 healed	0	NA	NA	0	0

PHASE 1 CLINICAL OBSERVATIONS DATA FOR DERMAL HD-DOSED ANIMALS (N=16)

Animal#	Dose Date	Obs Date	Site	Dose	Size LxW (mm)	Exudate	Erythema	Edema	Necrosis	Eschar	General Impression	Epithelialization	Inflammation	Granulation	Contraction	Infection	Rejection	Adherence	Durability	Vascularization
96-18-8	10/15/96	11/21/96	A	400 uL	12x18	0	NA	NA	NA	NA	NA	0	0	NA	NA	0	NA	NA	NA	NA
			B	400 uL	43x42	0	NA	NA	NA	NA	NA	0	0	NA	NA	0	NA	NA	NA	NA
			C	400 uL	40x34	0	NA	NA	NA	NA	NA	0	0	NA	NA	0	NA	NA	NA	NA
			D	400 uL	14x27	0	NA	NA	NA	NA	NA	0.5	0	NA	0.25	0	NA	NA	NA	0.5
			E	400 uL	30x23	0	NA	NA	NA	NA	NA	1.25	1	NA	NA	0	NA	NA	NA	0.5
			F	400 uL	12x36	0	NA	NA	NA	NA	NA	0	0	NA	1	0	NA	NA	NA	NA

ATTACHMENT C

Table C-1 Bandaging Technique

Summary Report 1 Sulfur-Mustard-Induced Full-Skin-Thickness Burns in Weanling Swine Model

Summary Report 2 Phase I, Part A, Debridement and Bandaging Techniques

Summary Report 3 Phase I, Part B, SM Application and Debridement Techniques

TABLE C-1. BANDAGING TECHNIQUE

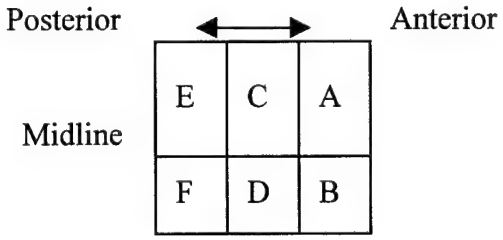
SM-Induced Lesion	Grafted SM-Induced Lesion
Dry Gauze Pads	Petroleum Impregnated Gauze
	Mineral Oil-Soaked Cotton
4X4 Dry Gauze	
Plaster of Paris Cast Mold	
Adhesive Telfa™ Pads	
Vetwrap™	
Stockingette	

Summary Report 1, Sulfur-Mustard-Induced Full-Skin-Thickness Burns in Weanling Swine Model
General Daily Experiment Preparation:

Pre-dosing Preparation: Within 24 hr of dosing, each animal was weighed. A chemical depilatory compound (Nair™ AG, Carter Products, Division of Carter-Wallace, Inc., NY, NY) applied to the ventral abdominal dosing area for approximately 7 min before rinsing. Minimal to no observable irritation the next day. Removal of Nair™ consisted of gentle washing with a 1:20 Ivory® dish-washing detergent (Proctor and Gamble, Cincinnati, Ohio) diluted with distilled water. Patting the area with warm water-soaked 4x4 gauze sponges (The Kendall Company Hospital Products, Boston, MA), followed by patting the area dry with gauze sponges or a clean towel. Food was removed approximately 8 hr before dosing.

Dosing Day Preparation (Day 0): Animals were anesthetized for SM exposure, wound biopsy and/or excision, and treatment application (autograft and Dermagraft-TC™ application). Atropine sulfate (AMVET Scientific Products, Yaphank, NY) was administered intramuscularly (IM) at 0.04 mg/kg body weight to control excessive salivation. Animals were anesthetized with a combination of xylazine (Ben Venue Laboratories, Inc., Bedford, OH) and Telazol® (Fort Dodge Laboratories, Inc, Fort Dodge, Iowa). Five mL of xylazine solution (100 mg/mL) were used to reconstitute Telazol® (250 mg tiletamine and 250 mg zolazepam) and the mixture dosed at 0.044 mL/kg BW IM. Tracheal intubation was performed and inhalation anesthesia was initiated using a concentration of 2.5 to 3 percent Isoflurane (Abbott Laboratories, North Chicago, IL) in oxygen at an initial flow rate of 2 L per min using an Anesco anesthetic machine (Anesco, Inc, Georgetown, KY) or Matrix anesthetic machine (Matrix Medical Inc., Orchard Park, NY). Anesthesia was maintained with an Isoflurane concentration of approximately 1-2.5 percent with flow rates reduced to a range of 800 mL to 1 L/min. Pretreatments were accomplished and/or dosing templates were applied once the animal was anesthetized. Following SM exposures or surgical application of grafts, the concentration of Isoflurane was reduced over time until the animal was on 100 percent oxygen, and then gradually changed to room air.

A six-site dosing grid (3 sites per side) was drawn on the ventral abdomen of each animal, as shown below.



Each site within the grid measured approximately 5 cm by 5 cm. An approximately 0.5-1 cm space separated each dosing square. The animal was placed in dorsal recumbency and the dosing grid was drawn approximately 1.5 cm lateral to and parallel to the teat line on both sides of the animal. Dosing templates were applied to each of the six sites prior to placing the animal in a

sling within a chemical fume hood. The animal was secured in the sling and allowed to stabilize for up to 20 minutes prior to dosing.
Each dosing template was constructed of a 5 by 5 cm square of duct tape applied to double-sided carpet tape of the same size. A 3-cm diameter circle was cut through the tape assembly, and between the two tapes a Whatman No. 2, microfiber glass filter paper (Whatman, Hillsboro, OR) was placed to cover the 3-cm diameter hole. A 3-cm diameter O-ring (Hewlett Packard, Wilmington, DE) was glued to the outer surface of the tape assembly and surrounded the 3-cm diameter hole.

SM Dosing: Sulfur mustard was supplied by USAMRICD. Purity, appropriate identification and storage condition information was provided by USAMRICD. SM samples were taken and evaluated by the MREF chemistry section to assure concentration. Appendix A contains chemistry analysis data. Samples were analyzed in triplicate by gas chromatography using a flame ionization detector (FID). In each case, the percent of the expected concentration was well within the acceptable ~ 20 percent range. Values ranged from 88.4 to 99.2 percent of expected.

A dosing volume of 400 µL SM was applied to each dosing template for a 2 hr exposure to produce the full-skin-thickness SM-induced dermal burns. SM was applied to the 3-cm diameter microfiber filter within the dosing template using a 500-µL glass Hamilton syringe (Hamilton Company, Reno, NV) with a blunt 18 ga needle. After application of SM, a size 3 teflon disc (Thomas Scientific Company, Swedesboro, NJ) was placed over the dosing site and a rubber stopper inserted in the dosing well to occlude the dose site, minimize SM evaporation, and achieve a uniform SM application. The rubber stoppers were secured on each side using a rubber tile float taped in place using Vetrap™ (3M). In Phase III, the 400 µL of SM was administered using a 1,000 µL Eppendorf pipet (VWR). Exposure times were reduced to 2 min and 3 min.

Decontamination Exposures: The animal was decontaminated by gently pressing a dry gauze sponge against each dose site for 30 sec to remove excess SM. A water-soaked gauze sponge was then gently pressed to each dose site for 30 sec. A water-soaked sports towel was placed over each side of the animal and a plastic-backed absorbent paper sheet was placed over the sports towel and held in place for 1 min. This step was repeated and then the area allowed to air dry overnight. After decontamination, the animal was allowed to recover from anesthesia, removed from the sling, extubated, and kept in the fume hood overnight in a modified animal transport crate. The next morning, the animal was anesthetized with the Telazol®/xylazine combination and an approximately 2-L plastic bag was placed over the entire dosed area. A relatively air-tight seal was formed using double-sided carpet tape cut in lengths to secure the bag to the area. A MINICAM™ (CSM Company, Birmingham, AL) was used to sample the air within this plastic bag after allowing it to equilibrate for approximately 15 min. The animal was removed from the hood, after acceptably low SM levels (≤ 0.5 TWA) were attained and returned to its home cage.

In Phase III, the decontamination procedure was changed to gently dabbing the dose sites with a dry sports towel for 20 sec. This removed the excess SM. A pilot study performed by the sponsor indicated that this procedure was as effective as the procedure described above.

Summary Report 2, Phase I, Part A, Debridement and Bandaging Techniques

ANIMALS 17-22 (96-24-8, 96-22-3, 96-28-12, 96-28-13, 96-30-5, and 96-31-10, respectively) Pretreatment and Dosing: Nair™ was applied for seven minutes to the dose site (ventral abdomen) the day prior to dosing. The dose volume was 400 µL with a 2 hr exposure. The dose sites for these animals were evaluated on Day 2, prior to excision.

Debridement and Bandaging Summary:

96-24-8 - Sites C, D, E, and F were excised using iris or iris-like scissors. An autograft was applied to sites C and D and Dermagraft-TC™ was applied to sites E and F. Sites A and B (contralateral) were not treated. Excision occurred along the plane of the mini-blisters (pockets of fluid) as demonstrated by LTC Janny for most of the sites. On D and F sites, subcutaneous tissue remained, but some excised areas were deeper than others. BANDAGE: Thermazine® was applied to lesions C, D, E, and F. Two dry gauze 4X4's were folded and placed over sites A and B. Vaseline-coated, 4X4 gauze (1 each) was wadded and placed over each graft site. On side A-C-E, two layers of two folded-in-half 4X4s were placed over the sites and a 4X8 adhesive Telfa™ pad placed over the sites using tissue adhesive bond (Skin Bond®,). This was thick and hard to secure (per technician and Study Director). On side B-D-F, the same was done, except that the folded-in-half 4X4s was only a single gauze and single layer. Vetrap™ was wrapped around the wound sites and a stockinette was placed over the pig and secured with surgical adhesive tape (1/2-in surgical tape wrapped around the chest and posterior abdomen). This animal was evaluated on day 2, 9, 16, 23, 30 ,and 37. Bandages were changed once per week until Day 23, when no further bandaging was performed. Bandaging was the same as described above, except on Day 16 two tongue depressors taped side-by-side were placed over the gauze padding inaddition to the method described above on both sides.

96-22-3 - Sites B and C were excised using iris scissors and the dermatome set at approximately 0.75 mm was used for sites A and D. Excision occurred along the plane of the mini-blisters (pockets of fluid) as demonstrated by LTC Janny for most of the sites. The chest caviety was too steep to remove damaged tissue on site A with the dermatome (less than 1/3 of the lesion removed), so the remainder of this lesion was surgically excised. An autograft was applied to sites A and B and Dermagraft-TC™ was applied to sites C and D. Sites E and F (contralateral) were not treated. On A, B, and C sites, subcutaneous tissue remained, but some excised areas were deeper than others. Site D dermatome skin was not a consistent thickness, but most of the lesion was removed. BANDAGE: Thermazine® was applied to lesions B and D only. Two dry 4X4 gauze were folded in quarters and placed over site E and 1 dry 4X4 gauze was folded in quarters and placed over site F. Xeroform™ 4X4 gauze (1 each) was wadded and placed over each graft site. On side A-C-E, two folded-in-half 4X4 gauze bandage were placed over the sites and two 4X8 adhesive Telfa™ pads secured the bandages using Skin Bond®. On side B-D-F, two folded-in-half 4X4 gauze were overlapped and a tongue depressor placed ontop and secured with the Telfa™ as described above. Surgical tape was used to secure the edges of the Telfa™ bandage. Vetrap™ and stockinette were not used. This animal was evaluated on day 2, 9, 16, 23, and 30. Bandages were changed once per week until day 23, when no further bandaging was performed. Bandaging changes for Day 9 were as follows : Thermazine® was applied to sites A and C. Dry, folded gauze was used for the no treatment sites, E and F. For sites A, C, and E,

two tongue depressors taped side-by-side were placed over the two folded 4X4 gauze pads. A single layer of 4X4 gauze pads were used on side BDF without the tongue depressor otherwise bandaging was as described above for both sides. Benzoin tincture was used on side BDF. Only a stockinette was placed on the animal. Bandaging on day 16 was as described on day 9. The animal was not bandaged after day 23.

96-28-12 - Sites B and E were excised using iris or iris-like scissors. Excision occurred along the plane of the mini-blisters (pockets of fluid) as demonstrated by LTC Janny for most of the sites. Sites A and F were excised using the dermatome set at the approximate 0.75 mm setting. An autograft was applied to sites E and F and Dermagraft-TC™ was applied to sites A and B. Sites C and D (contralateral) were not treated. Sites B and E were excised to the subcutaneous tissue. BANDAGE: Thermazine® was applied to lesions A and E. Two dry gauze 4X4's were folded and placed over site C and only a single dry 4X4 gauze was placed on site D. Xeroform™ gauze (1 each) was wadded and placed over each graft site. On side A-C-E, two layers of two folded-in-half 4X4 gauze were placed over the sites, followed by two tongue depressors taped side-by-side, and a 4X8 adhesive Telfa™ pad secured the bandaging using Skin Bond®. On side B-D-F, the same was done, except that no Thermazine® was used, single layers of single 4X4 gauze was placed directly over dose sites, and two taped side-by-side tongue depressors were used to secure the guaze and the remainder of the bandaging was the same. Vetrap™ was wrapped around the wound sites and a stockinette was placed over the pig and secured with surgical adhesive tape (1/2-in surgical tape wrapped around the chest and posterior abdomen). This animal was evaluated on day 2, 9, 16, 23, and 30. Bandages were changed once per week until day 23, when no further bandaging was performed. Bandaging was the same as described above, except on day 9. No Thermazine® was used on sites A, C, or E, Thermazine® was used on sites B and F. On day 16 bandaging was performed as described for day 2 except Thermazine® was applied to sites A and E and sites B and F were not treated with Thermazine®.

96-28-13 - Sites D and E were excised using iris or iris-like scissors. Excision occurred along the plane of the mini-blisters (pockets of fluid) as demonstrated by LTC Janny for most of the sites. Sites C and F were excised using the dermatome set at the 0.75 mm setting. Dermatome at the 0.75 mm setting were not consistant, largely due to technician training. An autograft was applied to sites C and D and Dermagraft-TC™ was applied to sites E and F. Sites A and B (contralateral) were not treated. BANDAGE: Thermazine® was applied to lesions D and F. Sites C, D, E, and F had a vaseline coated 4X4 gauze placed over each site. Sites A and B were protected using a dry 4X4 gauze. For side A-C-E, two dry 4X4 gauze were folded and overlapped over the dose sites. Two tongue depressors taped side-by-side were placed on top of the gauze and secured using two 4X8 adhesive Telfa™ pads adhered to the skin with Skin Bond®. On side B-D-F, the same was done, except that the folded-in-half 4X4 gauze was a single gauze in a single layer. Vetrap™ was wrapped around the wound sites and a stockinette was placed over the pig and secured with surgical adhesive tape (1/2-in surgical tape wrapped around the chest and posterior abdomen). This animal was evaluated on day 2, 9, 16, 23, and 30. Bandages were changed once a week as described above until day 23, when no further bandaging was performed.

96-30-5 - Sites A and D were excised using iris or iris-like scissors. Excision occurred along the plane of the mini-blisters (pockets of fluid) as demonstrated by LTC Janny for most of the sites.

An autograft was applied to sites A and B and Dermagraft-TC™ was applied to sites C and D. Sites B and C were excised using the dermatome set at the 1.0 mm setting. Dermatome sites at the 1 mm setting were the most consistent, largely due to the excision performed by an experienced technician. Sites E and F (contralateral) were not treated. BANDAGE: Thermazine® was applied to lesions A and C. No Thermazine® was used on side BDF. Vaseline-coated 4X4 gauze (1 each) was wadded and placed over each graft site. Sites E and F were not treated and dry 4X4 gauze was folded over each of these sites. For side A-C-E, a single 4X4 gauze was folded and layered over the lesions. Two tongue depressors taped side-by-side were placed over the gauze. Skin Bond® was spread around the A-C-E wound area to secure a 4X8 Telfa™ pad. On side B-D-F, two dry 4X4 gauze pads were folded and overlapped over the lesions. A 4X8 adhesive telfa pad was secured over the sites using Skin Bond®. Vetrap™ was wrapped around the wound sites and a stockinette was placed over the pig and secured with surgical adhesive tape (4-in surgical tape wrapped around the chest and posterior abdomen). This animal was evaluated on day 2, 9, 16, 23, 30 ,and 37. Bandages were once a week as described above until day 9, when Thermazine® was applied to sites B and D only. For both sides, two 4X4 gauze pads were placed over the vaseline-impregnated or dry gauze pads placed over the grafted or non-treated sites, respectively. Two tongue depressors taped side-by-side were placed over the gauze padding and the remainder of the method was performed as described above. Double gauze was used as this provided additional pressure to the grafted sites. Bandaging on day 16 was as described above. After day 23, bandaging was not continued.

96-31-10 - Sites B, D, and F were excised using a dermatome set at approximately 1.0 mm, and sites A, C, and E were excised using a Dremal Tool drum sander. An autograft was applied to sites C and D, and Dermagraft-TC™ was applied to sites A and B. Sites E and F (contralateral) were not treated. The dermatome areas were consistant and an approximately 1.0 mm thickness of skin was removed. Removal of the lesion using the Dremal Tool drum sander appeared to be less consistant and removed more tissue in some areas than desired. BANDAGE: Thermazine® was applied to lesions A, B, C, D, E, and F. Vaseline-coated, 4X4 gauze (1 each) was wadded and placed over each site. Both sides had two folded-in-half 4X4s overlapped over the sites and two tongue depressors taped side-by-side were placed over the gauze padding. A 4X8 adhesive Telfa™ pad secured the tongue depressors over the gauze using Skin Bond®. This was secured using 4-in adhesive surgical tape. Vetrap™ was wrapped around the wound sites. This animal was evaluated on day 2, 9, 16, 23, 30 ,and 37. Bandages were changed once a week as described above until stopped at day 16.

Summary Report 3, Phase I, Part B, Application and Debridement Techniques

ANIMALS 23-28 (97-50-14, 97-50-16, 97-60-11, 97-63-10, 97-1-7, and 97-1-4, respectively) Pretreatment and Dosing: Nair™ was applied for seven minutes to the dose site (ventral abdomen) the day prior to dosing. The dose volume was 400 µL with a 2 hr exposure. The dose sites for these animals were clinically evaluated on day 2, prior to debridement anf histologically evaluated after debridement on Day 2. Three of the lesions were debrided using the dermatome set at approximately 1.0 mm setting. Two biopsies were taken from each site for histopathologic evaluation after debridding the wound. One from the periphery and one from the center of the lesion. On day 7, the animal was euthanized and the sites excised for histopathologic evaluation. In addition, blood samples were collected for RBC and WBC counts with WBC differential counts on study day 0, 1, 3, and 7. Urine samples were collected twice a day for urinalysis and thiodiglycol determination beginning on study day 0 through study day 7.

Debridement and Bandaging Summary:

97-50-14 - Sites B, C, and F were debrided using a Zimmer dermatome set at ~1.0 mm depth. An 8 mm punch biopsy was taken from the periphery and central area of the lesion on day 2. Lesions were not bandaged. The animal was euthanized and the lesions excised on day 7.

97-50-16 - Sites A, D, and E were debrided using a Zimmer dermatome set at ~1.0 mm depth. An 8 mm punch biopsy was taken from the periphery and central area of the lesion on day 2. Lesions were not bandaged. The animal was euthanized and the lesions excised on day 7.

97-60-11 - Sites B, C, and F were debrided using a Zimmer dermatome set at ~1.0 mm depth. An 8 mm punch biopsy was taken from the periphery and central area of the lesion on day 2. Lesions were not bandaged. The animal was euthanized and the lesions excised on day 7.

97-63-10 - Sites A, D, and E were debrided using a Zimmer dermatome set at ~1.0 mm depth. An 8 mm punch biopsy was taken from the periphery and central area of the lesion on day 2. Lesions were not bandaged. The animal was euthanized and the lesions excised on day 7.

97-1-7 - Sites B, C, and F were debrided using a Zimmer dermatome set at ~1.0 mm depth. An 8 mm punch biopsy was taken from the periphery and central area of the lesion on day 2. Lesions were not bandaged. The animal was euthanized and the lesions excised on day 7.

97-1-4 - Sites A, D, and E were debrided using a Zimmer dermatome set at ~1.0 m depth. An 8 mm punch biopsy was taken from the periphery and central area of the lesion on day. Lesions were not bandaged. The animal was euthanized and the lesions excised on day 7.